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G. IVÁNOVICS

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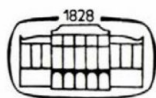
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INTERACTION OF HUMAN LYMPHOCYTES AND VIRUSES IN VITRO

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Interaction between phytohaemagglutinin (PHA) stimulated human lymphocytes and two DNA viruses (adenovirus type 5 and herpes simplex virus type 1) considered to be closely connected with lymphoid tissues has been studied. The fate of the same viruses was investigated also in non-stimulated separated lymphocytes for comparative purposes. To elicit this interaction infectivity titrations, immunofluorescent technique and electron microscopy were used. The production of viral antigens was investigated by complement fixation. It has been shown that in PHA-stimulated lymphocytes from peripheral blood of healthy donors adenovirus type 5 is capable to replicate in its infectious form. Prolongation of the interval between stimulation and infection of cells significantly influenced the dynamics of replication. Non-stimulated lymphocytes produced antigens but no infectious particles.

The important role played by leukocytes in the pathogenesis of viral diseases has been confirmed by many authors [1–9]. The function of leukocytes is not restricted to protection; in some cases they may carry and eventually even multiply certain viruses [8, 10–15]. Thus far only few members of the DNA virus group were found to be able to replicate in white blood cells from healthy donors, and even those only in leukocytes stimulated e.g. with phytohaemagglutinin (PHA) [16, 17]. Few such data are available concerning adenoviruses [18] in spite of the growing importance of this virus group for human pathology [19] and its close connection with the immune system. This has led us to investigate the connection between leukocytes and the latent adenovirus type 5 (Ad5). Experiments with herpes simplex virus type 1 (HSV-1) were also performed utilizing data published in connection with this virus [14].

Materials and Methods

Lymphocytes were separated from heparinized whole blood collected from healthy donors. Lymphocyte-rich supernatant plasma was obtained after sedimentation and subsequent filtration through a fibreglass column. Cells were sedimented (200 g, 5 min), washed in Parker's 199 solution and resuspended to a final concentration of 1.5×10^6 lymphocytes/ml in a medium composed of 25% autologous plasma and 75% Parker's 199 solution. PHA-P (Difco), when used, was added in a final concentration of 1 : 3000 [20, 21]. The Ad5 and HSV-1 were our own laboratory strains produced and titrated on HEp-2 tissue cultures. The suspension cultures of lymphocytes were infected mostly at their initiation with 0.4 CPD₅₀/cell Ad5 or 5×10^{-4} CPD₅₀/cell HSV-1. Cells were cultured usually for 5–6 days in

5% CO₂-air atmosphere at 37 °C. Apart from preliminary experiments cells were washed at 1.9 hr postinoculation in Parker's 199 solution (200 g, 5 min) and gently resuspended in a virus-free but otherwise unchanged medium.

Between 6 min and 154 hr after infection, samples were taken from the cultures at different intervals and the supernatant was separated from the cells by centrifugation (200 g, 5 min). Part of the cells from each sample was prepared for immunofluorescent and electron microscopic investigations, the remaining cell population was repeatedly washed and subsequently destroyed by 5 cycles of freeze-thawing. With HSV-1 no freeze-thawing was done.

Electron microscopy was performed by a JEOL JEM 100B electron microscope. Specimens fixed with glutaraldehyde and osmium tetroxide were embedded into Durcupan ACM, sectioned with LKB Ultratome 3 and contrasted with uranylacetate and lead citrate.

Immunofluorescence was observed by the modified method of COONS and KAPLAN [22, 23]. Both direct and indirect techniques, using rhodamine conjugated and fluorescein isothiocyanate conjugated globulins in cases of HSV-1 and Ad5, respectively, were applied.

Infectivity titrations were done for both viruses on HEp-2 tissue cultures according to REED and MUENCH [24] from both the supernatant and the destroyed lymphocytes.

Viral antigens from supernatant and destroyed cells were determined by a complement fixation micromethod (CF).

For statistical analysis, Student's *t*-test was used. In infectivity titrations (CPD₅₀-titres) parallel determinations could not be made because of the small volume of the specimens. Therefore, in these titrations the relative standard deviation of the method (S.D.M._{rel}) was determined by making 6 to 14 parallel titrations in different titre-ranges. The S.D.M._{rel} was generated on the basis of the following formula:

$$\text{S.D.M.}_{\text{rel}} = \sqrt{\frac{\sum_{i=1}^n \frac{Q_i}{(\bar{x}_i)^2}}{\sum_{i=1}^n (n_i - 1)}}$$

where n_i means the number of parallel titrations on the same sample, $\bar{x}_i$ the mean of these results, n is the number of samples titrated and

$$Q_i = \sum_{k=1}^{n_i} (x_k - \bar{x}_i)^2$$

If the difference between two data (in our case CPD₅₀-titres) exceeds 2 S.D.M._{rel}, it can be regarded as significant ($p < 0.05$). (Statistical analysis was done with a PDP 11/70 computer. Programs were developed and run by V. SCHRANZ.)

Results

In preliminary experiments where the medium was not changed for a virus-free solution at 1.9 hr after infection, the infectivity localized intracellularly was considerably increased by 16 hr in PHA-stimulated lymphocytes infected with Ad5, whereas no such change could be detected in non-stimulated lymphocytes infected with the same virus. In the supernatant, however, considerable infectivity remained throughout the experiment, and therefore it was impossible to exclude a somewhat accelerated uptake of virions by the lymphocytes at 16 hr, to account for the rise in infectivity at this sampling time. This has led us to change the medium at 1.9 hr (see Materials and methods) in all further experiments with both Ad5 and HSV-1.

In PHA-stimulated lymphocytes infected with Ad5 (Fig. 1) infectivity titration assays revealed a highly significant ($p < 0.01$) titre-rise intra-

cellularly at 20 hr after infection, followed by a second peak of intracellular infectivity at 93 hr. Similarly to the epithelial cells susceptible to Ad5, the virions were not released from the stimulated lymphocytes throughout the experiment. Correspondingly, after changing the medium for a virus-free one at 1.9 hr, the majority of infectivity was localized to the cells, the titres in the medium remaining very low.

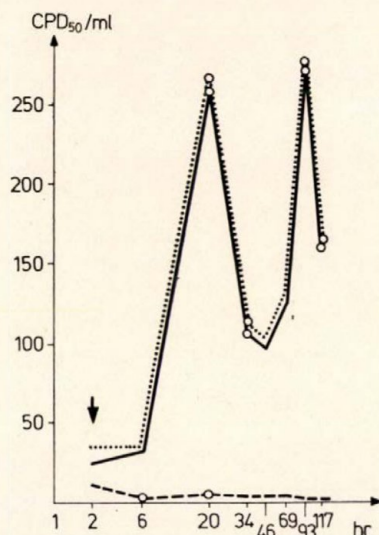


Fig. 1. Infectivity titres in PHA-stimulated lymphocyte cultures infected by adenovirus type 5. (Results of one out of four experiments with similar outcome.) Arrow: change of medium for a virus-free but otherwise unchanged solution. Intracellular (○—○): infectivity found intracellularly in 1 ml of the whole suspension culture. Extracellular (○---○): infectivity found in 1 ml of the supernatant after separation from lymphocytes. Total (○.....○): sum of the infectivity titres found intra- and extracellularly at the given point of time. Points symbolized with ○ represent values differing significantly ($p < 0.05$) from the previous value along the same line. For infectivity, $S.D.M._{rel}$ was 0.4199. Time points 2 hr before postinfection are not shown

In non-stimulated separated mononuclear cells no direct evidence of the production of infective virions could be found (Fig. 2). Between 0.5 and 1 hr postinfection (not shown in Fig. 2) an increase in intracellular and a decrease in extracellular infectivity occurred; both changes were significant and could probably be assigned to the adsorption of virions by lymphocytes.

The production of adenovirus antigens in stimulated lymphocytes was clearly demonstrated by CF (Fig. 3). The diagram indicating intracellular titres was similar to that representing infectivity, only the first peak appeared later, at 69 hr. In contrast to infectivity, a continuously rising titre of the supernatant was shown by CF. At the end of the experiment, at 154 hr, "total" values exceeded almost threefold the lowest ones obtained at 2 hr,

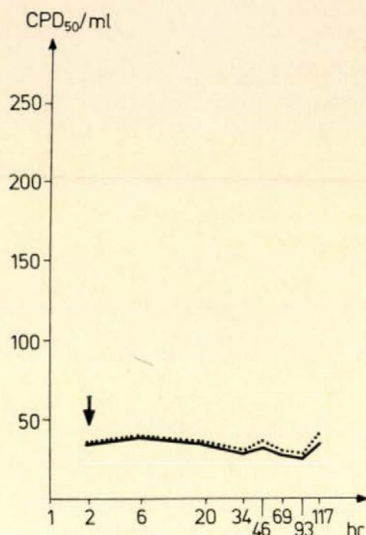


Fig. 2. Infectivity titres in non-stimulated lymphocyte cultures infected by adenovirus type 5. (Results of one out of four experiments with similar outcome.) For symbols, see legend to Fig. 1. Time points 2 hr before postinfection are not shown

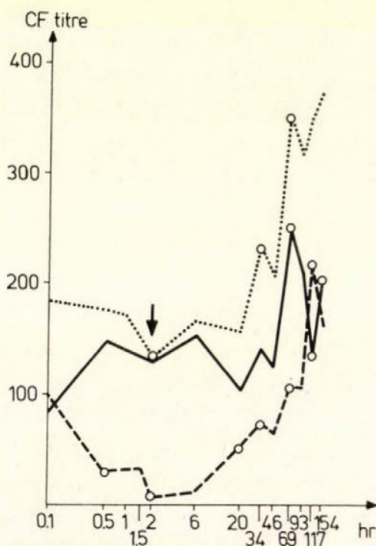


Fig. 3. Complement fixation titres in PHA-stimulated lymphocyte cultures infected by adenovirus type 5. (Results of one out of four experiments with similar outcome.) Arrow: change of the medium for virus-free solution. Points symbolized with $\circ$ represent values differing significantly from the previous value along the same line. For complement fixation, $S.D.M_{rel}$ was 0.4836. (For the determination of significance the t -test was used.) $\circ \cdots \cdots \circ$ total; $\circ - - - \circ$ extracellular; $\circ - - \circ$ intracellular

just after the change of the medium. The early significant decrease in the CF-titre of the supernatant at 0.5 hr coincided with the adsorption of virions.

In non-stimulated separated mononuclear cells, the CF showed a pattern strikingly similar to that of Ad5-infected PHA-stimulated lymphocytes, the only difference being that the peak of the intracellular activity occurred later, at 93 hr (Fig. 4). The "total" value at 154 hr exceeded 2.3 times the minimum read immediately after changing the medium.

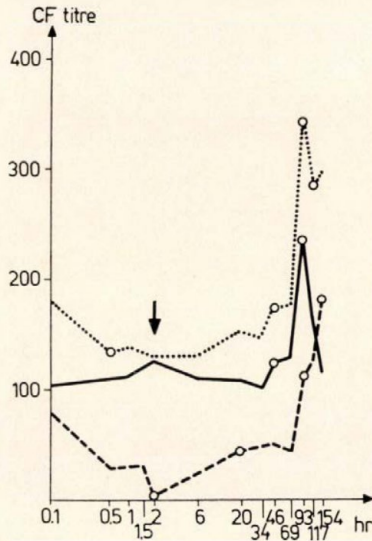


Fig. 4. Complement fixation titres in non-stimulated lymphocyte cultures infected by adenovirus type 5. (Results of one out of four experiments with similar outcome.) For symbols, see legend to Fig. 3

Production of infectious virus particles and viral antigens was significantly influenced by the length of the interval between stimulation with PHA and infection with Ad5 of the cells. When infection was done at maximum thymidine incorporation, i.e. 72 hr after the addition of PHA, the time necessary to reach the maximum of the infectivity and of the CF-titres was reduced to half of that required by the control.

The assay of interaction between Ad5 and PHA-stimulated lymphocytes by indirect immunofluorescence revealed a faint, ring-shaped specific fluorescence on the surface of about 1% of the cells as early as 6 min after infection. Subsequently the fluorescence became more intensive, more homogeneous, and the number of cells involved also increased. In samples taken at 69 hr or later, inclusion-like fluorescent formations appeared in the cell nuclei. In non-stimulated lymphocytes fluorescence showed the same pattern

with the maximum intensity appearing later, at 93 hr after infection, and no nuclear inclusions could be observed.

Electron microscopy revealed that virions had adsorbed to the cell membrane, invading the cytoplasm and the nuclei of the PHA-stimulated cells. At 20 hr, Ad5 virions showed a crystal-like arrangement in the nuclei of the stimulated blast cells, which is an accepted proof of active virus multiplication in the cell (Fig. 5).

In non-stimulated lymphocyte cultures, adsorption and penetration, but no viral replication could be observed. In one case, the donor lympho-

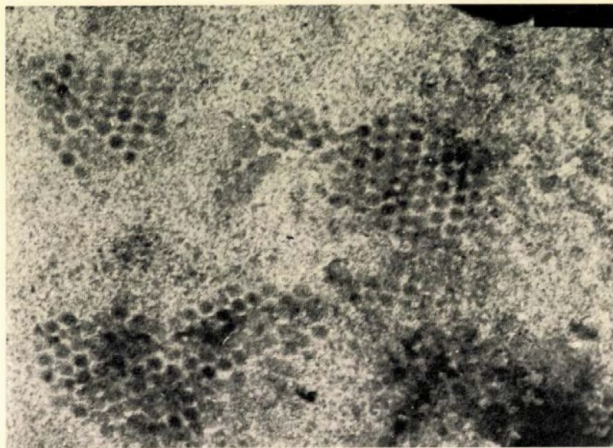


Fig. 5. Crystal-like grouping of adenovirus type 5 virions in the nucleus of a lymphoblast.
×40 000

cytes showed blast transformation (12% blast cells after 5 days of incubation) in the presence of Ad5 antigens. After 88 to 154 hr characteristic Ad5 crystals were detected in the nuclei of the blast cells from this donor.

Investigations of HSV-1 failed to disclose infectious virus growth either in stimulated or in non-stimulated lymphocytes, the lack of viral replication being probably due to the presence of heparin or to the low infecting dose. Complement fixing antigens were shown at low concentrations, while the presence of HSV-1 antigens in the cells was proved by immunofluorescence.

Discussion

The connection between adenoviruses and human leukocytes seems to be a neglected question. Latency of some types of human adenoviruses would, however, make it important to clarify their ability to multiply in leukocytes [18, 28].

Our results indicate that Ad5 is capable of replication in an infectious form in PHA-stimulated circulating human lymphocytes. Non-stimulated lymphocytes failed to support the multiplication of infectious particles, nevertheless the production of CF-antigens could be shown. A remarkable parallelism was found between the CF-titre and the intensity of indirect immunofluorescence. At the beginning of detectable fluorescence, at 6 min postinfection, only about 1% of the cells showed positivity both in PHA-stimulated and non-stimulated systems. Extrapolating these data, a hundred-fold increase of the infecting dose would lead to the infection of all available cells. This dose (40 CPD₅₀ per cell) is somewhat higher than that found by other authors for HeLa and KB cells [27-29]. This would mean that if our system were homogeneous, the lymphocytes needed a greater amount of virions adsorbed to produce infective particles. Our system, however, although enriched in T-cells by filtration through a fibreglass column, contained mixed cell populations and this makes it difficult to tell which of the cells are susceptible for Ad5 and which is the optimum infecting dose. Nevertheless, the differences between the data of stimulated and non-stimulated systems support the suspicion of T-cells being involved in the replication of Ad5, without excluding the similar role of other cell types. Therefore, the validity of our calculated infecting dose needs experimental support.

Ad5 behaved similarly in lymphoid cells as in Ad5-susceptible epithelial cells. The virions produced by the lymphocytes remained intracellular throughout the incubation period, and could be released only by destroying the cells (e.g. by freeze-thawing), while increasing amounts of CF-antigens in the supernatant pointed to the release of "soluble antigens" by the lymphocytes.

Our data indicate also that not only non-stimulated lymphocytes and lymphocytes in the first few minutes after contact with PHA but also PHA-lymphoblasts are susceptible to Ad5 adsorption and penetration.

By infectivity titrations it could be demonstrated that Ad5 replication was significantly influenced by the length of the interval between cell stimulation and cell infection.

Electron microscopy supported the results yielded by infectivity titrations: proof of viral replication was found in PHA-stimulated but not in non-stimulated cultures [30, 31]. The fact that Ad5 multiplication was detected in one of the non-stimulated cultures late after infection can be explained by the appearance of lymphoblasts shown to be able to produce Ad5 virions. As more time is needed for specific mitogens than for PHA to reach the optimum effect it is conceivable that somewhat more time elapsed until the crystals composed of virions had appeared in the nuclei. In this unplanned experiment the infection and specific stimulation (by one or more antigens of Ad5) of the cells led to the formation of lymphoblasts which in turn were able to produce Ad5 in its infective form. It follows that

PHA-lymphoblasts and blast cells resulting from specific stimulation may behave similarly concerning Ad5 replication.

It is well known that adenoviruses show an affinity to lymphoid cells, but the mechanism and consequences of that affinity remain to be clarified. It is also known that blast-type lymphoid cells may be continuously present in the organism, e.g. in the tonsils [18, 25, 26]. Our findings suggest such cells to be able to potentiate the persistence and perhaps the replication, too, of this virus. On the other hand, the increasing application of more efficient immunosuppressive drugs may create conditions suitable for the activation of the virus, as has been shown in the rat [32]. Considering the fact that this rather widespread human virus group includes latent and oncogenic members as well, a more detailed inquiry into the relationship between the immune system and the infecting agent seems to be justified.

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COMPARISON OF AGAR GEL PRECIPITATION AND COMPLEMENT FIXATION TEST FOR THE DETECTION OF BRUCELLA OVIS INFECTION

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Blood samples from 710 sheep raised in 20 different farms were examined for *Brucella ovis* antibodies. Thirty samples were positive with complement fixation, 35 samples with precipitation, and 58 samples with both tests. It has been concluded that the gel precipitation technique is adequate for screening but for individual testing both methods should be used simultaneously.

Infective epididymitis and orchitis of rams associated with *Brucella ovis* were described in 1953 by BUDDLE and BOYES [1]. Since then the disease has been reported from all over the world. In Hungary, SZABÓ and NYIREDY [2] were the first to describe its occurrence and to confirm that the causative agent was identical in features to *B. ovis* strain Weybridge 63/290 of BUDDLE and BOYES [1, 3]. The disease has become known by veterinary practitioners and a number of organ, semen and blood samples are being sent to our laboratory. However, verified cases represent only a fraction of the infections; serological studies [4] have shown that manifest disease and infection may occur in seemingly healthy animals.

Although the clinical symptoms and autopsy findings are characteristic, in the detection of infected animals serological examination plays the decisive role. The complement fixation test is widely used [3, 5–24]. CLAPP *et al.* [11] elaborated a method for decreasing the anticomplementary effect of the antigen. BIBERSTEIN and MCGOWAN [6] used sonicated bacterial cells as antigen and showed that the results correlated more closely with other tests than those obtained with heated bacterial suspension. Some authors [1, 14, 17, 25] used heated and sonicated polysaccharide antigen, others [2, 5–8, 18] employed only the sonicated proteinic antigen.

The antigenic structure of *B. ovis* strains was examined by gel precipitation methods [25–27] using heated and sonicated bacteria and antigens prepared by treatment with acetone, ether and other chemicals. For routine examination MATTHEW and TRUEBLOOD [28] used tube precipitation and agar gel diffusion techniques.

The double diffusion technique was applied by MEINERSHAGEN *et al.* [17], but they found that the method was less sensitive than the complement fixation test. In view of its simplicity and specificity [24], it seemed, however, desirable to re-investigate the value of this method for screening.

BIBERSTEIN *et al.* [6, 7] have shown that the frozen proteinic complement fixing antigen rapidly loses its titre but not its specificity. As we have been using a similar, proteinic complement fixing antigen prepared from living bacteria by sonication, introduction of the agar gel diffusion technique offered a less laborious and less expensive routine procedure.

Materials and methods

Blood samples were taken from 710 rams and ewes kept in 20 different farms.

Antigen for complement fixation and agar gel precipitation test was prepared as described by BIBERSTEIN *et al.* [6, 7].

Complement fixation test was performed at 1 : 5 dilution by the method used in brucella serodiagnosis [29]. Haemolysis under 50% was regarded as a positive reaction.

Gel precipitation test. Difco Bacto agar (2% for coating, 0.9% for diffusion medium) in pH 7.0 phosphate buffer saline was used. Each slide was covered with 4.5 ml diffusion medium. Undiluted and inactivated sera were used at 0.1 ml amounts. The slides were incubated in moist chamber at room temperature and read after 48 hr.

Results

Comparison of results obtained with complement fixation and precipitation tests (Fig. 1) is presented in Tables I and II. As control, all blood samples were examined with *B. abortus* and *B. melitensis* antigens; these tests were uniformly negative.

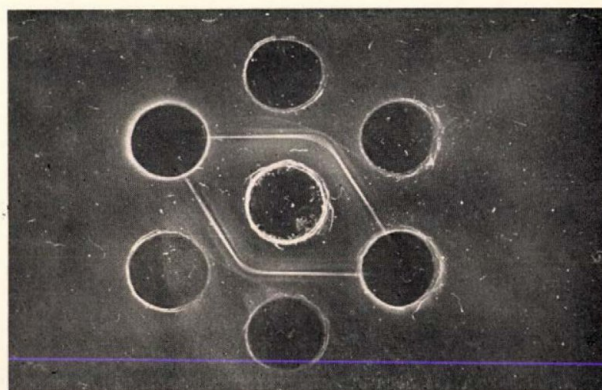


Fig. 1. Precipitation test with *B. ovis*

Table I

Positive, negative and doubtful titres for B. ovis antigen in sheep serum samples examined with complement fixation and precipitation test

	Complement fixation test			Precipitation test		Total
	negative	positive	doubtful	negative	positive	
Number	590	88	32	608	102	710
Per cent	83.1	12.3	4.5	85.6	14.4	100.0

Table II

Distribution of positive, negative and doubtful titres for B. ovis antigen in sheep serum samples examined with complement fixation and precipitation tests

Total number of blood samples, 710

	Number	Per cent
Precipitation +	58	8.2
Complement +		
Precipitation +	35	4.9
Complement —		
Precipitation —	30	4.2
Complement +		
Precipitation +	9	1.2
Complement ±		
Precipitation —	23	3.2
Complement ±		
Precipitation —	555	78.2
Complement —		

Discussion

With complement fixation test 12.3%, with precipitation test 14.3% of the sheep blood samples were positive. This finding shows the reliability of the latter method. If doubtful results of the complement fixation test are considered of diagnostic value, the precipitation technique is less sensitive. Different results of the two tests for individual sera do not lessen the value of either method, since the level of "complement fixing" and "precipitating" immunoglobulins may vary in the individual sera. It is known that after infection first "precipitating" antibodies appear and this is followed by a rise in "complement fixing" antibodies.

B. ovis infection of rams is easily diagnosed by clinical examination and autopsy. The infection of ewes and lambs is less easy to detect. Simultaneous application of the complement fixation and precipitation tests yields undoubtedly the best results. However, because of its simplicity and its

inexpensiveness as compared to the complement fixation test, the precipitation technique may be recommended for screening. For individual examinations, both tests should be used simultaneously.

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TWO- AND THREE-DIMENSIONAL HEXON CRYSTALS

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Soluble hexon of type 1 adenovirus was highly purified with different techniques and dialysed against 0.5 M acetate buffer. With this procedure tetrahedral crystals were produced from the soluble hexon capsomers of the virus capsid. Electron microscopic observation of the crystallization process revealed the development of dense two-dimensional "crystal sheets" following dialysis, in homogeneous hexon preparations containing single hexons. No such formations were observed so far with other types. The occurrence of two-dimensional crystals decreased proportionally to the appearance of three-dimensional crystals, which refers to their possible role in the mechanism of three-dimensional crystal formation.

The icosahedral adenovirus capsid consists of 252 capsomers (protein subunits), of which the 240 situated on the edges and faces of the icosahedron are called hexons and the remaining 12 located on the vertices of the virion are called pentons [1]. The penton consists of a vertex capsomer called penton base and a projection called fibre. In the intact type 5 adenovirion, the hexons display a polygonal structure 9.5 nm in diameter and an inner hole 2.5 nm in diameter [2]. In our studies of the soluble hexons of type 1 adenovirus, similar holey structures were observed and using the freeze-fracture technique the hexon forming three polypeptide molecules were clearly distinguished (unpublished data). After high-grade purification of the type 1 adenovirus hexons and determination of their parameters, amino acid composition and isoelectric point [3], the virus specific protein was produced in crystal form and changes occurring in the course of the crystallization process in hexon preparations were studied in the electron microscope.

Material and methods

Soluble hexon of type 1 adenovirus was prepared and purified with a technique described earlier [4]. Protein concentration was determined according to LOWRY *et al.* [5] in Pye Unicam SP 800 spectrophotometer.

For crystallization the purified hexon preparation was dialysed against 0.5 M acetate buffer pH 4.5 at 4 °C for three days without stirring. Subsequently the samples were stored at 4 °C in test tubes.

Serial electron microscopic examinations were performed to follow the crystallization process. Samples were negatively stained with uranyl acetate and studied in a JEM 100 B electron microscope at 60 kV.

Results

Hexon preparations used for the crystallization procedure and purified by chromatography and gel filtration were found to contain large amounts of homogeneous, diffusely scattered hexon capsomers. No regular array could be revealed among them, neither in electron microscopic preparations made directly after separation nor in those prepared after storage at 4 °C for several months. Serial electron microscopic examinations performed during and after the acetate buffer dialysis displayed a regular array of hexons. Small but regular hexon groups appeared first, while later they became arranged in two-dimensional crystal sheets of several hundred nm in size (Fig. 1). The hexons were situated in this network in a single layer, organized densely in regular rows. Their relation was similar to the hexons situated on the faces of the isocahedral virion. Every second row was shifted 4.75 nm along the

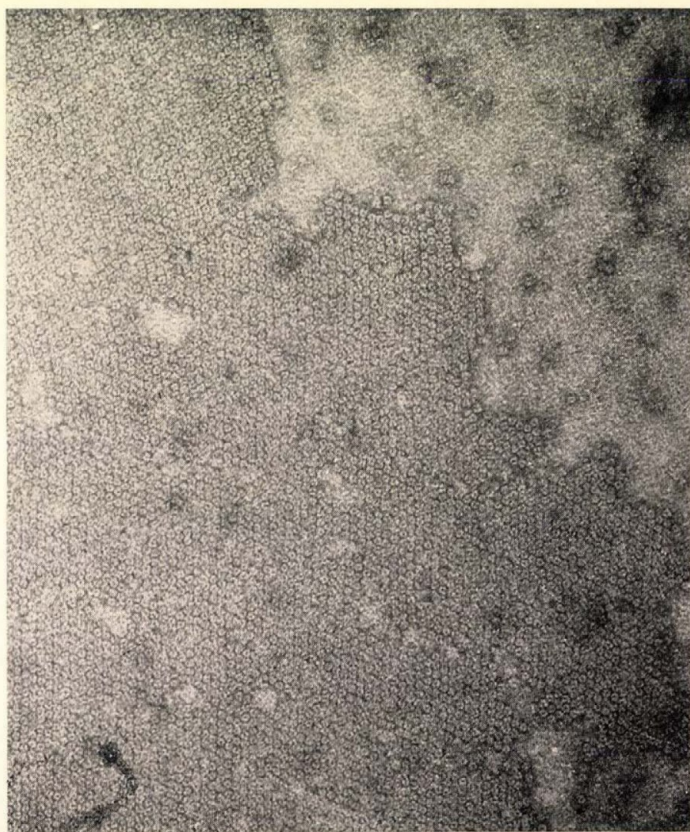


Fig. 1. Two-dimensional crystal sheet after crystallizing dialysis. Hexons organized densely in regular rows in a single layer. $\times 150\,000$

longitudinal axis, this distance being about half the diameter of one hexon particle. Each hexon has two nearest neighbours not only in its own row but also in the two next hexon rows. Consequently each hexon has 6 nearest neighbours according to the hexagonal structure [6-8]. Not only large two-dimensional crystal sheets but also smaller structures developing probably from the disruption of a larger sheet could be detected. At the sites of disruption the partly irregular lines of break were fitting (Fig. 2). According to the structure of the crystal network, hexon aggregates with angles of 60° - 120° developed.

Crystallizing different hexon preparations of 1-6 mg/ml protein concentration, slight deviations were observed in the size of the individual crystals. The edge length of the tetrahedral crystals was found to be 0.01-0.03 mm under the light microscope (Fig. 3). The crystals were fragile and even the use of a cover slip made them split into small fragments. The crystals



Fig. 2. Disrupted two-dimensional crystal sheet displaying 60° and 120° disruption. Lines of break fit together at several sites. $\times 90\,000$

could be sedimented by low speed centrifugation and washed with 0.5 M acetate buffer. In this buffer they were stable and failed to show any change either at 4 °C or at room temperature for a long period. The crystals dissolved quickly in 0.04 M Tris buffer pH 8.2.

After dissolving the crystals under the light microscope, the antigenic characteristics of their proteins were demonstrated in gel diffusion. The crystal solution and the initial hexon preparation gave in the presence of antihexon serum confluent precipitation lines, which proved their antigenic

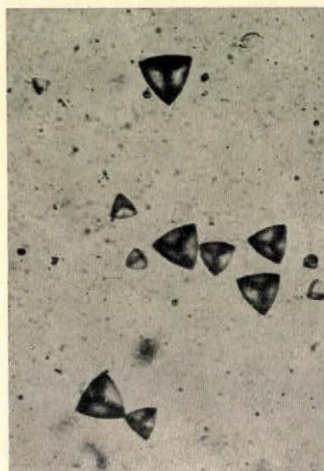


Fig. 3. Tetrahedral hexon crystals. $\times 190$

identity. No precipitation line was obtained either with the mother liquor or with the washing solution of the crystals. This indicates partly a significant decrease of the dissolved protein content during the crystallization process, partly the fact that the crystals do not dissolve in the course of acetate buffer washing. The electron microscopic picture of the crystal solution and the initial material was similar. Hexons were present in large numbers without displaying any systemic array. According to the results of gel-diffusion, the hexon capsomers kept their original antigenicity during the crystallization and redissolution process in spite of the high ion concentration and the low pH. Simultaneously with the appearance and increase of the tetrahedral crystals observed under the light microscope, the number of the two-dimensional crystal sheets decreased, as revealed by the electron microscope. Beside tetrahedral three-dimensional crystals and two-dimensional crystal sheets, microcrystals with a regular linear inner structure (Fig. 4) were seen; their size ranged between 100 and 260 nm.

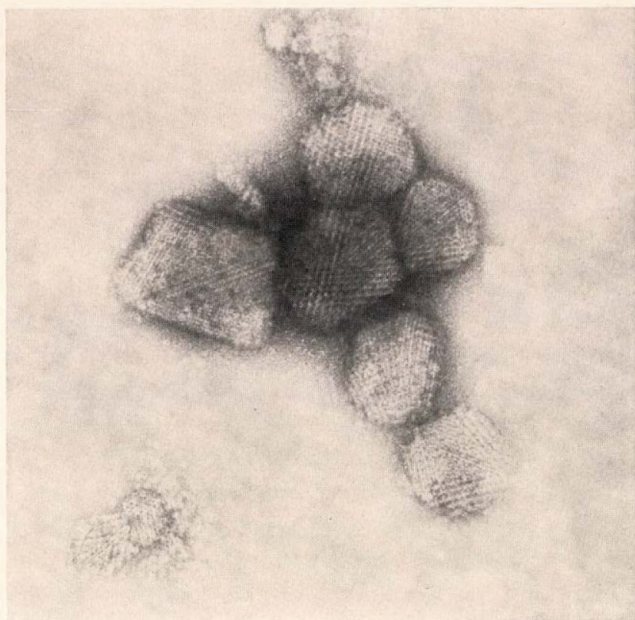


Fig. 4. Multilayered microcrystal with linear, regular inner structure. $\times 120\,000$

Discussion

PEREIRA and WRIGLEY [9] reported on the two-dimensional crystal lattice in type 5 adenovirus preparations; when reconstructing the capsid from separated nonamers *in vitro*, they detected the crystal sheet of holey lattice structure. Similar crystal lattices were found also by CARSTENS and MARUSYK [10] who attempted to crystallize the hexon from a separated but not purified protein mixture of the same virus. Studying the type 1 adenovirus, we too detected similar two-dimensional holey crystal lattices but of a different structure; it seemed to have been a crystal defect [11].

The present results suggest that in the course of dialysis at low pH and high ion concentration it is the dense two-dimensional crystal sheet which develops first, providing a basis for the final three-dimensional crystal. This was supported by the fact that the number of two-dimensional crystal sheets increased during the crystallization process until the appearance of three-dimensional crystals, then it decreased until only a few sheets had remained. This array could only be observed during acetate buffer dialysis. Neither 0.01 M phosphate buffer (pH 7.0) dialysis nor storage for several months was seen to induce a regular symmetric aggregation.

At neutral pH, charged protein molecules lose their charge in the pH range corresponding to the isoelectric point, displaying there the lowest solubility. A shift to both acid and alkaline direction increases the solubility and so does a low ion concentration [12]. This might be the explanation for the optimum range of crystal production being between pH 3.5 and 4.5 [13-15], in good agreement with the isoelectric point of the hexons being pI 4.7 [16] for type 2 and pI 4.55 for type 1 [3].

Though hexons constituting the adenovirus capsid are identical in morphology and antigenic characteristics and no difference could be revealed among them by chromatography, they display differences in their isoelectric point. SHORTRIDGE and BIDDLE [16] found a pI 3.1 for peripentonal hexons, whereas 3.7 for the hexons situated at the edges; pI 4.7 and 4.55 characterized only hexons located in the middle of the triangular faces. Hexons at the edges and faces and those situated peripentonally show different bonds in the virion capsid and different virion building polypeptides can be found among the pentons and peripentonal hexons and among the hexons of the triangular faces [17]. One may assume that the hexon population purified chromatographically fails to be homogeneous and that besides the hexons of the faces, peripentonal hexons different in isoelectric point and bonding relations might also be present. The latter can produce the holey hexon lattice or the occasionally detected tubular formations which might be due to their slightly different nature [11]. Proper hexons building up the dense two-dimensional crystal sheet display the same bondage relations by which they constitute the virion capsid. To support this assumption, further studies are, however, necessary.

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TOXOPLASMA ANTIBODIES IN OPHTHALMOLOGICAL PATIENTS

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Sera of 532 patients suffering from different ophthalmological diseases were studied for the presence of toxoplasma antibodies. Complement fixation test was positive in 44.9% of the patients. As compared with the 26.8% positivity rate determined earlier in the normal population the seropositivity in eye-patients was significantly higher. The highest incidence, 50%, was found in the age group of 21–30 years. In diseases with a confirmed aetiological role of toxoplasmosis, the incidence of seropositivity was higher than in the total material. This suggests that *Toxoplasma gondii* may have had a role in part of the diseases of the patients studied.

The clinical picture of congenital eye toxoplasmosis is characteristic and has been well known for several decades, whereas the existence of contracted eye toxoplasmosis has been questioned for long; the symptoms in adult age were interpreted as an activation of the congenital disease. In contrast with the earlier view that congenital eye toxoplasmosis fails to show a characteristic picture, in the last 15 years a number of clinical, serological and parasitological evidences has indicated its aetiological role in diseases of the posterior and anterior segment of the eye [1–7]. Case reports as well as extensive statistical evaluations indicate clearly the connection between toxoplasmosis and certain ophthalmological conditions, especially acute granulomatous chorioretinitis appearing around the macula and the large veins [8–15].

To confirm the clinical diagnosis of toxoplasmosis in patients of two ophthalmological departments serological examinations were carried out for more than 5 years.

Material and methods

Toxoplasma antigen. Soluble antigen was prepared by the technique of JIRA and BOZDECH [16] from the peritoneal exudate of mice infected with the RH strain of *Toxoplasma gondii*.

Human sera. Patient serum was inactivated after arrival at 56 °C for 30 min and stored in the frozen state until use.

Complement fixation reactions (CFR). Examinations were carried out in Takátsy's Microtiterator by Kolmer's cold fixation. The initial serum dilution was 1:10. Haemolysin and complement were the products of the Institute for Serobacteriological Production and Research Human, Budapest. Human sera giving high titres in earlier studies were used as controls.

Results

The presence of toxoplasma antibodies was studied with CFR in the sera of 532 randomly chosen eye-patients. Sera with titres 1:10 or higher were considered positive. Data concerning age, sex, clinical diagnosis and results are presented in the Figures and Table I.

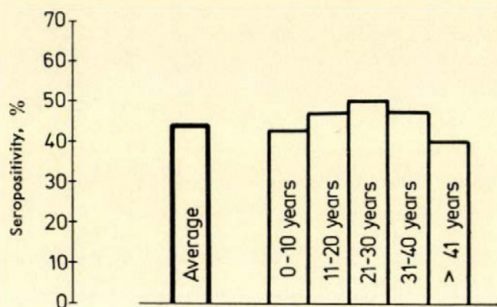


Fig. 1. Seropositivity of eye-patients according to age

Figure 1 demonstrates the seropositivity of eye-patients according to age. Of the 532 sera studied 44.9% were positive. Analysing the seropositive cases according to age, the positivity rate was found to increase until the age of 21-30 years (50%) and to decrease over 31 years.

The occurrence of seropositivity according to sex and age is presented in Fig. 2. No significant sex differences could be observed but in the 0-10 years age group the males showed a higher positivity rate (48.2%) than the females (38.1%), and the difference was even more striking in the age group of 11-20 years, where the male patients showed 56.5% positivity in contrast with 41.4% for the females.

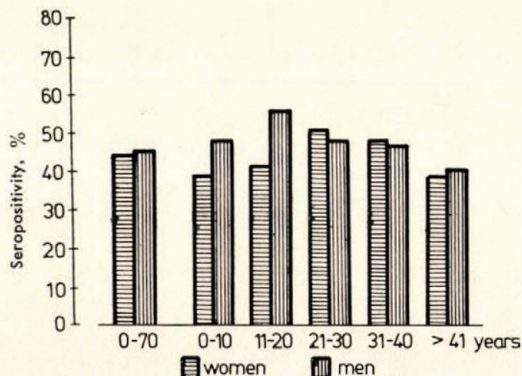


Fig. 2. Incidence of seropositivity according to age and sex

Table I

Percentage distribution of positive cases according to titre

CFR titre	Percentage of positive cases
1 : 10	18
1 : 20	32
1 : 40	27
1 : 80	17
1 : 160	4
1 : 320	0.8
1 : 640	1.2

The distribution of positive cases according to the titres are presented in Table I; this shows that 77% of the positive cases had titres of 1 : 10 to 1 : 40, whereas only 23% showed a titre of 1 : 80 or higher.

Figure 3 shows the incidence of seropositivity in the whole material as well as according to diagnoses. The seropositivity rate in cases eventually connected with toxoplasmosis is presented in a separate column. Patients

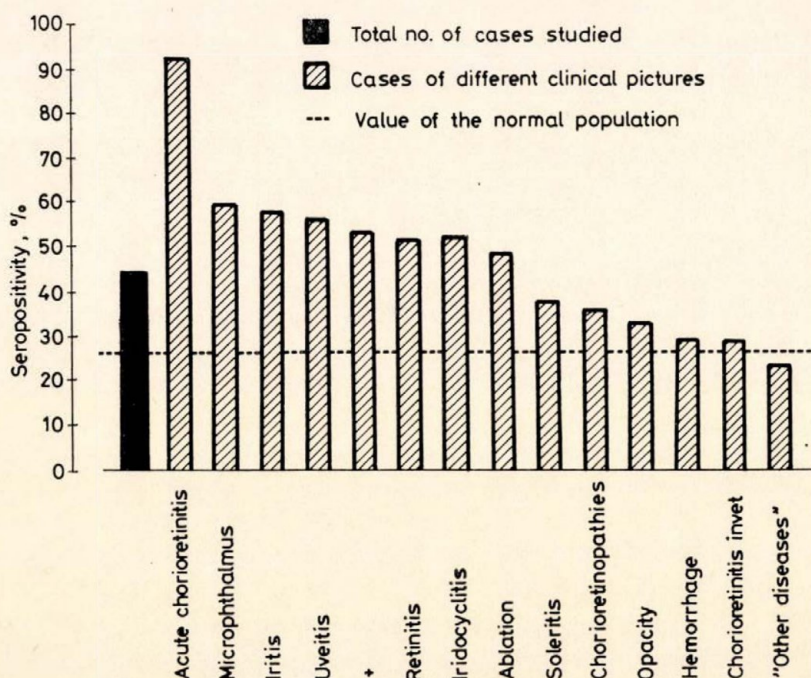


Fig. 3. Incidence of seropositivity in different clinical pictures. + = symptom-free mothers whose children were examined for congenital eye anomalies

whose disease has not so far been shown to be connected aetiologically with toxoplasmosis, formed the group of "other diseases". Of the 532 sera, 44.9% were positive as compared with the 26.8% positivity rate (dotted line) determined earlier for the normal population [17]. In diseases whose aetiological relationship with toxoplasmosis is well known, the incidence of seropositivity was significantly higher than the average for the other patients. The highest (92%) incidence was found in patients with acute chorioretinitis, though as compared with the seropositivity rate for all the cases studied, a significantly increased rate was obtained also in patients with microphthalmus, iritis and uveitis. Seropositivity in the group of "other diseases" was almost the same as among the normal population.

Discussion

The possibility of contracted toxoplasma infection of the eye has been first raised by WILDER [18] who found 41 toxoplasmosis cases in patients 16–72 of age whose eyes were enucleated. In 31 of the 41 patients only the eyes showed symptoms and chorioretinitis was observed in each case. In paraffin and celloidin section the protozoons were clearly observed. This observation was then confirmed experimentally, inducing similar changes by inoculating strains of low virulence into the eye chamber of rabbits [18]. Similar experimental evidence was obtained by NIEBROJ *et al.* [19] who observed panophthalmitis after intrabulbar inoculation in 24 rabbits. The retina displayed necrotic features, and in the uvea an inflammatory process took place with cellular infiltration. Similar results were reported by TABBARA *et al.* [20] and HIGAKI [21]. In the opinion of DESMONTS [22], toxoplasmosis has an important role in the induction of posterior uveitis. Recurrences were frequent in children above 10 years of age, in juveniles and young adults and were infrequent over 40 years. PASMANIK and ATIAS [23] diagnosed among 100 ophthalmological patients 27 congenital and 29 contracted toxoplasmosis cases which were confirmed also serologically. SEEMAN and KLENKA [9] examined the sera of 351 patients with diagnosed uveitis; the antibody level was significantly increased in 145 patients. CRAWFORD [24] found the whole retinal substance filled with toxoplasma cysts in an eye which was removed because of retino-chorioiditis recurring for 9 years. OLURIN *et al.* [25] studied the sera of 131 patients with chorioretinitis and of 90 healthy controls, with the Sabin–Feldman reaction. They found 95% positivity in the chorioretinitis group in contrast with 72% of the control group.

In our experiments the sera of 532 random eye-patients were studied with CFR for the presence of toxoplasma antibodies. As compared to the

26.8% positivity found earlier in the normal population, a 44.9% positivity was found in the ophthalmological material. As in the normal population, the positivity rate rose parallel with age and was highest (50%) in the 21 to 30 years age group. In agreement with the data in the literature [22], over 40 years the seropositivity rate was lower than the average for ophthalmological patients. As to the sex distribution, in the 0–10 and 11–20 years age groups the positivity was 10% and 15% higher in males, respectively. This is in contrast with the data published by JIRA and BOZDECH [16] and DESMONTS [22] who observed a slightly higher incidence of positivity in females.

Analysing our results according to the clinical picture in cases where the aetiological role of toxoplasmosis is common, the seropositivity rate was significantly higher than the average for eye-patients. The 92% seropositivity rate observed in patients with acute chorioretinitis was in good agreement with the findings of JIRA and BOZDECH [16] who found 88% positivity in patients suffering from juxta-papillary chorioretinitis, whereas GUIGOF [26] observed only 28–39% positivity by CFR in patients suffering from the same disease. In our material 77% of the titres ranged from 1 : 10 to 1 : 40. This tallies with data in the literature which indicate that the serum titre of patients with ophthalmological diseases caused by toxoplasmosis were low, except in acute infections or activated chronic processes [8, 11, 23]. Our results justify the assumption that *Toxoplasma gondii* has an aetiological role in the eye diseases of patients showing seropositivity.

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DIANHYDRODULCITOL TREATMENT OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION IN SUCKLING MICE

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Mice 2–4 days of age were pretreated with a single 5 mg/kg dose of dianhydrodulcitol (DAD) and later infected intracerebrally with lymphocytic choriomeningitis (LCM) virus. These animals had a lower mortality rate and died later than the untreated control animals. Thus DAD pretreatment prevented in part of the animals the development of lethal meningitis, the consequence of LCM virus infection, reducing the cellular immune response. This effect of DAD could equally be observed in animals infected at the age of 16–18 days and of 4 weeks.

The immune response of the animal determines the course of intracerebral (i. cer.) LCM virus infection. Interventions resulting in immune depression prevent the development of lethal meningitis following i. cer. LCM virus infection [1]. In adult animals, the immune depressive effect of chronic treatment with the lymphotropic cytostatic agent, dianhydrodulcitol (DAD), prevented the lymphocytic choriomeningitis induced by i. cer. virus infection in 50% of the animals [2]. In newborn and suckling mice, a single dose of DAD ineffective for adult mice, was found to cause lymphoid atrophy and lethal wasting [3]. In the present experiments the course of i. cer. LCM infection was studied in suckling mice subjected to DAD treatment at the age of 2–4 days and to virus infection at the age of 2 and 4 weeks.

Materials and methods

Experimental animals. CFLP mice of both sexes were used.

Dianhydrodulcitol, the epoxid of unitolactor, a cytostatic agent of the alkylating group, was kindly supplied by Chinoin Chemical and Pharmaceutical Works Ltd., Budapest. The substance was dissolved in distilled water and used within 30 min.

LCM virus infection. The W. E. strain was maintained in our Institute in serial mouse brain passages and stored at -70°C . Phosphate buffered saline (PBS) solution pH 7.0 was applied for the preparation of brain suspensions and virus dilutions. The virus was titrated in young adult mice, inoculating them i. cer. with 100 LD₅₀ (in 0.03 ml volume) of the virus. Neurological symptoms characteristic of lymphocytic choriomeningitis (tremor, convulsion) were controlled twice every day throughout the experiment and death of the animals was registered.

Reisolation of virus. Mouse groups were inoculated i. cer. at the end of the experiments with a 1:10 dilution of the brain suspension of each surviving animal. Reisolation of the virus was detected on the basis of the characteristic symptoms and death of the animals.

Study of the lymphoid system. (a) Absolute lymphocyte count was determined in blood samples from the caudal vein under standardized conditions. (b) To determine the relative lymph organ weight, the body weight of each sacrificed animal was determined and after dissection the spleen and thymus weights were determined as well.

$$\text{Average relative spleen weight} = \frac{\text{average spleen weight, mg}}{\text{average body weight, g}}$$

$$\text{Average relative thymus weight} = \frac{\text{average thymus weight, mg}}{\text{average body weight, g}}$$

(c) Determination of lymph organ index:

$$\text{Spleen index} = \frac{\text{average relative spleen weight in DAD treated group}}{\text{average relative spleen weight in control group}}$$

$$\text{Thymus index} = \frac{\text{average relative thymus weight in DAD treated group}}{\text{average relative thymus weight in control group}}$$

Statistical evaluation. From the average values for the individual groups ($\bar{x}$) and the standard errors or the averages ($\pm S\bar{x}$) comparative evaluations were carried out using Student's t test. The level of significance was $p = 0.05$.

Results

Thirty-two litters of 2–4 days old mice were subjected to intraperitoneal (i.p.) DAD treatment. The number of animals was reduced to 8–10 in each litter and half of the animals received 5 mg/kg DAD on a single occasion. The other half was given PBS solution. The incidence of deaths was registered daily and body weight determinations were performed weekly for 35 days after DAD treatment. The number of litters studied, average body weights and deaths are presented in Fig. 1a. The DAD treated animals showed a considerable lag in weight development as compared to their sibling controls. Thirteen percent of the animals died within 15 days following DAD treatment. They were wasted, their body weight being 2–4 g lower than the average weight of the animals still alive. Two percent of the untreated control siblings died before the 10th day; later no further deaths occurred.

To study the effect of DAD treatment on the lymphoid system, the absolute lymphocyte count was determined on the 12th day and the animals of 2 litters were sacrificed on the 14th day and those of 3 litters on the 24th and 35th days. Their relative lymph organ weight and lymph organ index were determined. After DAD treatment the absolute lymphocyte count was significantly lower on the 12th day in these than in the untreated animals. The average relative thymus weight and spleen weight were significantly lower on the 14th and 24th days, whereas no essential differences from the controls were observed on the 35th day (Fig. 1b). LCM virus infection with 100 LD₅₀ was carried out 14 days after DAD treatment in the mice of 4 litters, at the age of 16–18 days (Experiment 1) and at 24 days, thus in 26–28

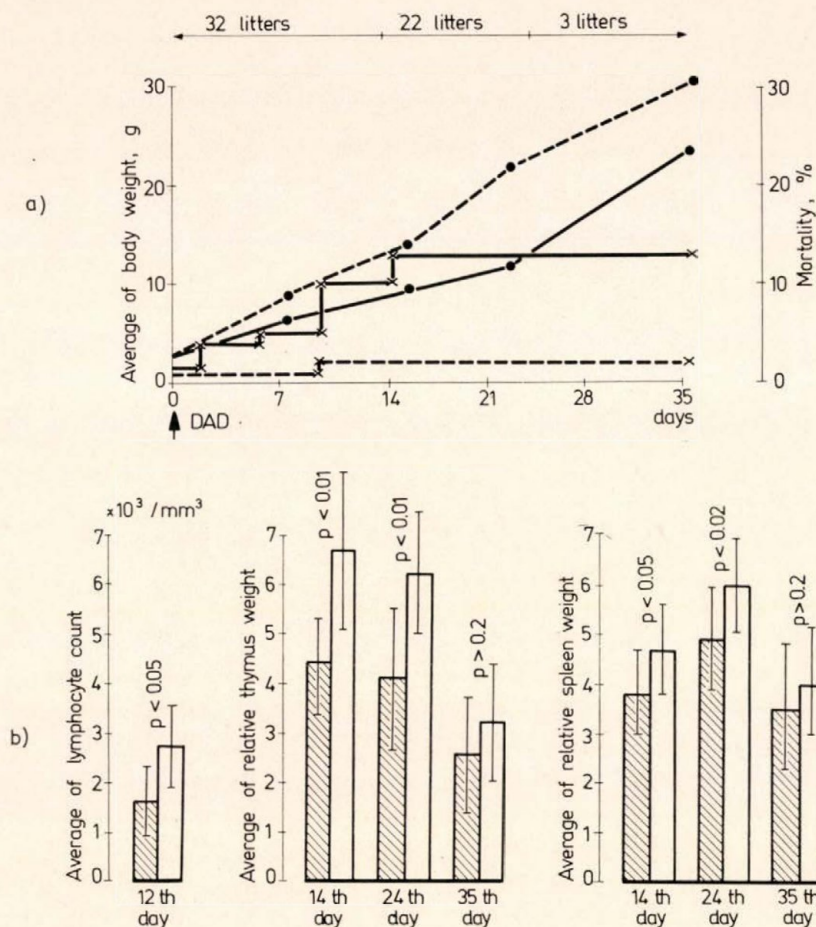


Fig. 1. Lethality, average body weight (a) and lymphoid organ data (b) of DAD treated mice. Body weight: ●—● DAD, ●—● control. Lethality: ×—× DAD, ×—× control. Shaded columns: DAD; open columns: control

days old animals of 8 litters (Experiment 2). DAD treated 16–18 days old animals formed the DAD-LCM-I group and 26–28 days old animals the DAD-LCM-II group. The PBS treated siblings constituted the corresponding C-LCM-I and C-LCM-II groups. Simultaneously with the LCM virus infections, animals of 4 and 8 litters were inoculated with the brain suspension of uninfected mice. Of these animals the DAD treated 16–18 days old ones formed the DAD-I, the 26–28 days old animals the DAD-II and their corresponding PBS treated siblings the C-I and C-II groups. Mouse groups and treatments applied in the individual groups are presented in Table I. Both experiments were terminated 21 days after virus infection. Fig. 2 demonstrates the rate and time course of deaths in the observation period following virus infection.

Table I
Mouse groups and their treatment

Experiment	Group	Number of mice	I. P. treatment at 2-4 days of age	I. cer. inoculation	Age at infection, days
I	DAD-LCM-I	18	DAD	LCM virus	16-18
	C-LCM-I	18	PBS	LCM virus	16-18
	DAD-I	18	DAD	×	16-18
	C-I	18	PBS	×	16-18
II	DAD-LCM-II	32	DAD	LCM virus	26-28
	C-LCM-II	32	PBS	LCM virus	26-28
	DAD-II	32	DAD	×	26-28
	C-II	32	PBS	×	26-28

× = Uninfected mouse brain suspension.

Of the virus infected suckling mice, 66% of the C-LCM-I group died 7-10 days after virus infection. Of the suckling DAD-LCM-I group only 33% died in the period between the 10th and 17th day postinfection.

Four weeks old animals belonging to the C-LCM-II group died 6-10 days after virus infection, displaying neurological symptoms. In the DAD-LCM-II group the daily death rate was lower and 10% of the animals survived without neurological symptoms.

Thus, in the DAD treated groups the mortality rate was lower and deaths took place later than in the control groups. Virus could be reisolated from the brain of each surviving animal of the infected group. No death occurred in the uninfected groups.

Discussion

A cellular immune reaction constitutes the basis of the lethal choriomeningitis developing after i. cer. LCM virus infection. Consequently the course of virus infection depends on the immune response of the animals and so also on their age [4]. In adult animals with intact immune system the infection causes a lethal choriomeningitis while newborn mice do not develop meningitis. They survive the infection and become virus carriers. After infection of suckling mice, the rate and time of death depend on the age of the animals at the time of infection. The number of surviving animals decreases with age and animals infected in adult age succumb earlier than young ones [5, 6].

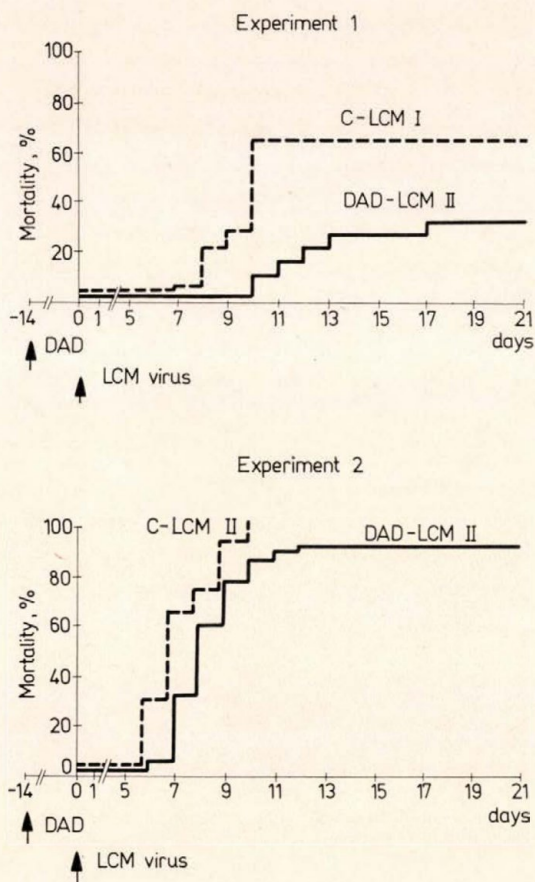


Fig. 2. Rate and time of deaths after LCM virus infection

In good agreement with these observations, in the present study the mice not pretreated with DAD and infected with LCM virus at the age of 4 weeks (C-LCM-II) all died, while only 66% died among the suckling mice (C-LCM-I). In our earlier study [3] mice given a single dose of 5 mg/kg of DAD at the age of 2–4 days died either with wasting or survived the treatment but became underdeveloped, displaying lymphoid atrophy. I. cer. LCM virus infection too showed a different course. The mortality rate was lower in DAD pretreated mice than in the untreated controls. DAD pretreatment prevented thus in part of the animals the development of lethal meningitis, exerting an immune suppressive effect. This effect of DAD treatment could be observed in animals infected at 16–18 days as well as 4 weeks of age, but the effect was different. The mortality rate after LCM virus infection decreased upon DAD pretreatment to 50% in animals infected at an early age, whereas in animals infected at the age of 4 weeks the decrease was only 10%. The differ-

ence may be ascribed not only to the age dependence of the state of the lymphoid system at the time of infection but also to its regeneration after DAD treatment. Data of Fig. 1b suggest namely that while the atrophy of lymph organs still persisted at 24 days after DAD treatment, on the 35th day it had again become normal.

The present results suggest that similarly to the consequences of neonatal thymectomy [7], a single DAD treatment applied at the age of 2–4 days causes not only wasting and lymphoid atrophy but also reduces the immune response.

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DETECTION OF ANTIBODIES TO CYTOMEGALOVIRUS, HERPES SIMPLEX VIRUS AND RUBELLAVIRUS WITH THE MICRO-ENZYME IMMUNOASSAY*

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Serum samples of pregnant women (200), non-pregnant women (100) and children (36) were tested for the presence of antibodies to cytomegalovirus (CMV), herpes simplex virus (HSV) and rubellavirus with the micro-enzyme immunoassay (micro-EIA), the indirect immunofluorescence antibody (IFA) technique and the haemagglutination-inhibition (HI) test. Micro-EIA gave the highest positivity rate: 78% for CMV, 86% for HSV and 85% for rubellavirus compared to 67% (CMV) and 84% (HSV) of the IFA technique and 79% (rubellavirus) of the HI test, respectively. Among IFA and HI positive sera the occurrence of micro-EIA negative ones was 6% for CMV, 4% for HSV and 9% for rubellavirus. It is concluded that the introduction of micro-EIA will improve the diagnostic and screening work of virus laboratories.

For the detection of antibodies to viruses in human sera several well standardized *in vitro* methods are being used. Among them, the complement-fixation reaction, haemagglutination-inhibition test, neutralization of viral cytopathic effect and immunofluorescence technique are predominantly applied for the quantitation of humoral antibodies. The technique applied depends mainly on the aim of the study and the virus involved. These serological methods seemed to meet most of the requirements of the antibody determination.

Recently, however, the enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) [1, 2] promise a new, extremely sensitive, rapid and specific method for detecting antibodies. Since automatization of EIA performed on polystyrene plates (micro-EIA) [3, 4] is a simple procedure, we decided to apply the method for determining the presence of some virus antibodies in sera collected from pregnant women within the scope of our Family Planning Project [5].

Before introducing micro-EIA for large scale screening, we compared its efficacy and specificity with that of the haemagglutination-inhibition (HI) and the indirect immunofluorescence antibody (IFA) tests. This comparison is the basis of the present report.

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Materials and methods

Serum samples were selected from our serum bank set up to clarify the role of viruses in fetal malformations. In the present study, 200 samples from pregnant women, 100 sera from non-pregnant women wearing intrauterine devices, and 36 sera from children aged between 1 and 5 years were tested. Sera were kept at -20°C until use.

For HI test and micro-EIA, antigens were purchased from Flow Laboratories (Irvine, Scotland), while for the IFA technique, cells infected with herpes simplex virus (HSV) or cytomegalovirus (CMV) were used.

HEp-2 cells grown in minimal essential medium were completed with 10% calf sera and human embryonic fibroblast (HEF) cells derived by trypsinization from 3-month-old fetuses and grown in Parker 199 medium supplemented with 10% calf serum.

HIL strain of HSV type 1 and AD-169 of CMV were used.

HI test was performed according to Flow Manual using heparin- MnCl_2 treatment of sera for eliminating non-specific inhibitors.

A modified version [6] of the IFA technique described by LEINIKKI [7] was applied.

Conjugate. Anti-human IgG (Hyland Laboratories, List No. 017352) was conjugated with alkaline phosphatase (Sigma, Type VII) as described by VOLLER *et al.* [8].

Micro-EIA. The standard technique [8, 9] was used with minor modifications. Linbro polystyrene plates (Cat. No. 76-321-05) were sensitized separately with the following complement fixing antigens: rubella (Cat. No. 40-827-41), HSV (Cat. No. 40-607-44) and CMV (Cat. No. 40-613-44). Of 1 : 200 dilution of the antigen in coating buffer 200 μl were added to each well. After an overnight incubation at 4°C , plates were emptied and washed three times with PBS-Tween. Then 150 μl of serum per well were added, the plates were incubated at 30°C for 2 hr, washed and 150 μl of the conjugate diluted in PBS-Tween containing 40 g/l bovine serum albumin (Fluka AG, No. 05480) were added to the wells. After repeated incubation and washing, 150 μl of the substrate (Sigma Tablets, No. 104-105) from a dilution of 1 g/l in 10% diethanolamine buffer were added. Substrate reaction time at 30°C was 60 min for HSV and CMV, and 90 min for rubellavirus. The reaction was stopped by adding 50 μl of 3 mol/l NaOH to each well. Negative and positive sera were tested on every plate and a blank section (well without serum) was also set up.

Evaluation of micro-EIA. For titration of antigens, conjugate, positive and negative sera, colour (extinction) developed in each well was measured by a Beckman spectro-colorimeter at 400 nm. When single dilutions (1 : 100) of serum samples were tested, the plates were read first with the naked eye recording the obvious positive ones by their yellow colour and then the "negatives" were measured with the photometer.

Results

1. *Extinction of positive and negative sera.* After determining the optimal dilutions of the antigens (1 : 200) and the conjugate (1 : 700), 3 negative and 3 positive sera were titrated for each virus. Their CMV and HSV antibody content was determined by the IFA test, to rubellavirus by the HI test. Figure 1 shows the results. On the basis of these data a 1 : 100 dilution of the serum samples was tested for comparison. Serum samples having an extinction value above 0.20 for CMV and HSV antibodies and 0.15 for rubellavirus antibodies were considered positive.

Specificity of micro-EIA was controlled by a blocking test, absorbing 3 HSV positive sera with the HSV, and 3 CMV positive sera with the CMV antigens. Ten μl serum were layered on HSV- and CMV-infected and non-infected cells. After 3 hr incubation at room temperature, they were collected, diluted and tested. Due to absorption, extinctions fell from the positive range to the negative one (Table I).

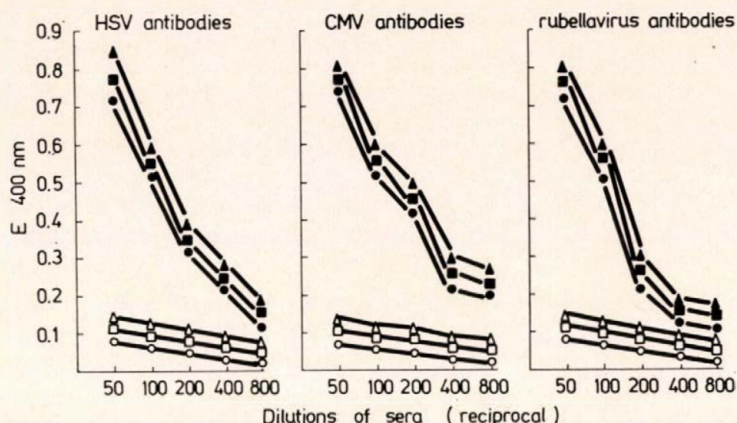


Fig. 1. Titration of positive and negative sera by micro-EIA. Full symbols stand for positive, empty ones for negative sera

Table I

Extinction of serum samples before and after absorption

Serum sample	HSV		CMV	
	before	after	before	after
No. 1	0.30	0.11	0.40	0.17
No. 2	0.34	0.11	0.36	0.16
No. 3	0.46	0.14	0.52	0.18

2. *Antibodies in the serum of pregnant women, non-pregnant women and children.* Altogether 336 serum samples were tested simultaneously; micro-EIA was compared with the HI test for rubellavirus antibodies, and with the IFA test for CMV and HSV antibodies. For screening purposes one serum dilution was tested: 1 : 10 with HI and IFA tests and 1 : 100 with the micro-EIA. Data are summarized in Table II.

Less negative sera were detected by the micro-EIA than by the other two methods; this reflected the higher sensitivity of the micro-EIA. Especially antibodies to CMV were detected more frequently with the micro-EIA than with the IFA technique. The greatest difference between the two tests was found in the group of children, where of the 36 sera tested 6 were positive in the IFA test and 17 with the micro-EIA.

Regarding antibodies to HSV, there was a close correlation between the results obtained by the IFA test and the micro-EIA. In every group the difference in the percentage of positive sera was within 4% (Table III).

With rubellavirus antibodies, the rate of positivity was slightly higher with the micro-EIA than with the HI test. Several micro-EIA negative sera

Table II*Occurrence of micro-EIA negative and positive sera according to IFA and HI titres*

Source of sera	No. of sera tested	Titre*	CMV		HSV		Rubellavirus	
			micro-EIA		micro-EIA		micro-EIA	
			negative	positive	negative	positive	negative	positive
Pregnant women	200	<10	34	24	13	17	19	25
		≥10	11	131	10	160	19	137
Non-pregnant women	100	<10	14	11	3	—	—	11
		≥10	1	74	2	95	3	86
Children	36	<10	13	17	20	1	8	8
		≥10	2	4	—	15	2	18
Total	336	<10	61	52	36	18	27	44
		≥10	14	209	12	270	24	241

* Concerns IFA for CMV and HSV, and HI for rubellavirus antibodies.

Table III*Percentage of positive sera*

Group	Antibodies to					
	CMV		HSV		Rubellavirus	
	IFA	micro-EIA	IFA	micro-EIA	HI	micro-EIA
Pregnant women	71	78	85	89	78	81
Non-pregnant women	75	85	97	95	89	97
Children	17	58	42	44	56	72
Total	67	78	84	86	79	85

(24 in number) could, however, be detected among the 265 sera positive with the HI test. Of the 24 samples 4 were negative with the micro-EIA despite their titres having been 1 : 128 with the HI test. On repeated testing of the samples, the same high titres were obtained with the HI test while no antibody could be detected with the micro-EIA. Thus, technical errors could not be responsible for the discrepancies.

3. *Efficacy of the micro-EIA.* Apart from controls (positive and negative sera, blanks) 85 to 90 samples may be tested on one plate. According to our experience, one technician can handle 10 plates, approximately 900 sera in two days. Dilution of sera and sensitization of plates with the antigen(s) are done on one day, and on the next day the rest of the procedure is per-

formed. Reading ten plates with the naked eye takes about 1 hr. Usually, 20 to 25% of the wells have to be evaluated photometrically and this takes about 2 hr. With the IFA technique, even when cells containing the antigens are available, not more than 30 slides (8 samples per slide) can be stained daily. Therefore, the micro-EIA is a considerably faster procedure than the IFA technique and its speed can further be improved by complete automatization of the evaluation [3, 4].

Discussion

For an adult population, the results obtained with the micro-EIA run parallel with those of the IFA and the HI tests. The higher positivity rate of antibodies detected with the micro-EIA in sera of children for CMV and rubellavirus may partly be due to the still existing maternal IgG acquired transplacentally. The sensitive micro-EIA may detect the persisting maternal IgG in sera of children under 5 years. Clarification of this possibility needs, however, further experiments as no such a higher positivity rate was found in the case of HSV antibodies.

Among sera positive for antibodies either with the IFA or the HI test, the micro-EIA showed some negatives. Their number was 6% for CMV (14 of 223), 4% for HSV (12 of 282) and 9% (24 of 265) for rubellavirus antibodies. The sera positive only with the IFA and the HI tests were retested and titrated. All the 14 samples for CMV and the 12 for HSV antibodies were positive with the IFA test at 1 : 10 dilution. Of 24 samples positive for rubellavirus antibodies, 4 displayed a high titre ($\geq 1 : 128$). Since the HI test measures IgG as well as IgM antibodies, the presence of the latter may explain why they were positive only with the HI test. On the other hand, VOLLER and BIDWELL [10] did not find such discrepancies when investigating sera with the micro-EIA. They only found 2 micro-EIA negative sera among the 20 with an HI titre between 1 : 16 and 1 : 32. Variations of the avidity of antibodies to the antigens used in the tests might perhaps be responsible for occasional deviations.

Despite the fact that no serological method is qualified to measure the true value of an antiserum [11], we believe that by using standard reagents, national or even international standardization of the micro-EIA can be achieved.

In laboratories where automatization of the assay with a simple device [4] is not yet available, visual reading [12] should be applied to spot obvious positive samples. With a combination of visual and photometric evaluation the micro-EIA is a rapid, economic method. Taking into account its high sensitivity [1] and specificity [13, 14] it is the technique of choice for large scale screening.

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CELLS CONTAINING EPSTEIN–BARR NUCLEAR ANTIGEN (EBNA) IN PERIPHERAL BLOOD*

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The anti-complement immunofluorescence (ACIF) method was used to demonstrate EBNA in B lymphocytes separated from the peripheral blood of patients with infectious mononucleosis (IM) or other diseases. In the acute phase of IM of Epstein–Barr virus (EBV) origin, 0.5–1% of the B lymphocytes proved to be positive in 6 of the 8 patients tested. In two of the positive cases the test was repeated 20 and 26 days, respectively, after clinical symptoms had appeared; the result was negative in both cases. No EBNA-positive cells were found in the peripheral blood of 17 patients with Hodgkin disease, 3 with systemic lupus erythematosus and 2 with lymphosarcoma. It is supposed that EBNA-positive cells appear in detectable amount exclusively in the acute phase of EBV infection.

The Epstein–Barr virus (EBV) nuclear antigen (EBNA) is invariably detectable in cells bearing the EBV genome, both in cultured cells and in those obtained by biopsy [1–3]. In the heterophile positive cases of infectious mononucleosis (IM) the causal role of EBV has been proved by HENLE *et al.* [4] and confirmed by several authors including ourselves [5].

The relationship between EBV and lymphocytes has raised interesting questions; among others, the presence or absence of EBNA-positive cells in IM is still unsettled. EPSTEIN [6] assumes that in IM patients the viral genome is carried by some unknown cells in a latent form, i.e., without expression of EBNA or any other virus-induced antigen. In contrast, KLEIN *et al.* [7] detected EBNA in 0.5–2% of B lymphocytes from the peripheral blood of patients suffering from IM.

We have adopted a simple reliable method to demonstrate whether there are EBNA-positive cells in the peripheral blood in IM and in other diseases, such as Hodgkin disease, in which very high anti-EBV titres have been observed [8, 9], systemic lupus erythematosus (SLE) and lymphosarcoma, in which too, high anti-EBNA titres have been reported [10].

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Materials and methods

Smear preparations from peripheral B lymphocytes. Lymphocytes were separated on Ficoll-Uromiro gradient from heparinized blood taken several hours before. The method, illustrated in Fig. 1, was described by JONDAL and KLEIN [11] and slightly modified by us. Ficoll-Uromiro has a specific weight of 1077 mg/cm^3 ; it is composed of 24 parts of 9% Ficoll Pharmacia and 10 parts of Uromiro Bracco.

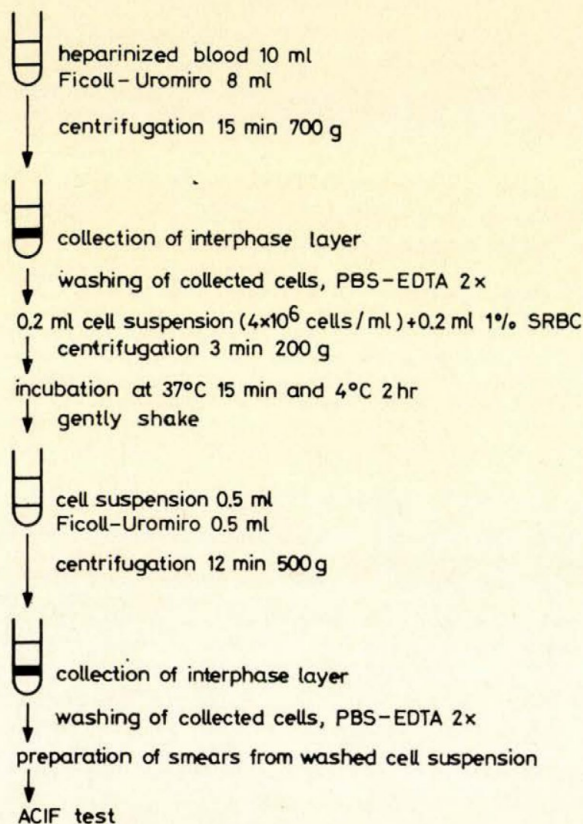


Fig. 1. Method for detection of EBNA-positive cells in peripheral blood

Separated lymphocytes were washed in a Ca^{2+} - and Mg^{2+} -free phosphate buffer of pH 7.4 containing 0.05% EDTA (PBS-EDTA). The procedure aimed at removing macrophages was omitted. The majority of the T cells, the presence of which would have disturbed detection of EBNA-positive cells, was removed as described by JONDAL [12]. The cell suspension rich in B cells was washed twice in PBS-EDTA and smears prepared from it were fixed in ice-cold acetone-methanol (1 : 1). In Giemsa-stained smears most of the cells appeared as homogeneous lymphocytes.

Demonstration of EBNA-positive cells by the anti-complement immunofluorescence (ACIF) method. The method described by REEDMAN and KLEIN [1] was used. Fixed smears were covered by a mixture of equal volumes of PBS [1] and anti-EBV-positive fresh serum containing both anti-EBNA and complement. The preparations were incubated for 30 min at 37 °C and thoroughly rinsed in distilled water. Then 1 : 20-diluted FITC-labelled anti-human complement (Hyland) was dropped on the preparations, which were then re-incubated at 37 °C for 30 min, thoroughly rinsed with distilled water and dried. Control preparations

were treated (a) with anti-EBV-negative serum and (b) with FITC-labelled anti-human complement without human serum. Buffered glycerol was placed on the dried preparations, which were then covered with a coverslip. A Leitz Orthoplan UV microscope was used at $500\times$ magnification. Each smear was thoroughly examined and each percentage value was computed from at least 1000 cells.

EBV-VCA-IgG and EBV-IgM antibodies were determined as described elsewhere [5].

Results

We failed to detect EBNA-positive cells among the lymphocytes examined without separation because such smears showed an intensive fluorescence even if FITC-labelled anti-human complement was added without human serum. Removal of the majority of T cells resulted in well evaluable preparations; evaluation was not disturbed by the few macrophages present in the smears.

Table I

Detection of EBNA-positive cells in the peripheral blood of heterophil-positive IM patients

Patients	Days after first symptoms	Anti-EBV IgM titre	EBNA-positive cells in purified B lymphocyte fraction, per cent
S. Gy. ♂	7	1 : 20	0.5
V. A. ♀	10	1 : 20	1
B. A. ♂	10	1 : 40	1
	20	1 : 20	0
E. L. ♂	8	1 : 20	0.5
	26	1 : 20	0
B. T. ♂	11	1 : 40	0
B. I. ♀	9	1 : 40	0.5
F. K. ♀	12	1 : 40	0.5
F. L. ♂	8	1 : 20	0

Eight cases of IM were examined for EBNA-positive cells. A case was accepted as acute EBV infection if anti-EBV IgM was demonstrated in a serum sample. In two cases, a second blood sample was tested after the first had proved EBNA-positive. EBNA-positive cells were detected in the peripheral blood of six of the eight patients; 0.5 to 1.0% of the cells in the B-lymphocyte-rich preparations displayed specific fluorescence. In fact, this indicates a much lower percentage, for B lymphocytes represent less than 1% of white blood cells. In the second samples no EBNA-positive cells could be detected.

No EBNA-positive cells were demonstrated in peripheral blood samples taken from patients suffering from Hodgkin disease, SLE or lymphosarcoma. In the serum samples of the same patients anti-EBV showed high titres (between 1 : 40 and 1 : 640 in Hodgkin disease and between 1 : 80 and 1 : 160 in SLE and lymphosarcoma), but EBV-IgM antibody, indicative of acute EBV infection, could not be detected.

Discussion

In the light of literary data and our present observations, it is clear that EBNA-positive cells appear and are demonstrable in the peripheral blood of patients in the acute phase of IM. This is in accordance with the results published by KLEIN *et al.* [7], but does not support the view [6] that cells bearing the EBV genome persist without any detectable manifestation of viral antigen in the peripheral blood during IM.

RICKINSON *et al.* [13] assume that the lymphoblastoid cell lines growing continually from IM *in vitro* are transformed by EBV in two steps, i.e., these cell lines would not originate from *in vivo* transformed (therefore EBNA-positive) cells; the EBNA-positive cells would represent only the few cells in which the virus cycle had stopped at a certain phase.

The presence in the peripheral blood of EBNA-positive cells and cells cytotoxic for EBV-transformed cells [14] suggests, though does not prove, that B lymphocytes are transformed into EBNA-positive cells *in vivo* and that the host's response accounts for the disappearance of the EBNA-positive cells.

In peripheral blood, EBNA-positive cells cannot be detected by the procedure applied by us, except in blood samples obtained during the acute phase of EBV infection. In other diseases (Hodgkin disease, SLE), the high antibody titres point to an earlier EBV infection. In this respect it is of interest that anti-EBNA first appears as late as 2–4 months after infection [15]. Presumably, the antigenic stimulus of the circulating EBNA-positive cells is not sufficient to induce their production; greater numbers of EBNA-positive cells must be present elsewhere (e.g. in lymph nodes) to supply the adequate stimulus. From this respect the EBNA-positive cells observed by VELTRI *et al.* [16] in human tonsils during exudative tonsillitis deserve attention. These authors assume that tonsils may represent reservoirs of cells carrying the EBV genome. Owing to the control by cell-mediated immunity [17], any condition (e.g., Hodgkin disease, SLE) in which cellular immunity is damaged may lead to an increase in the number of cells carrying the EBV genome and, consequently, to an increased antibody response. Eventually, one should reckon with an enhanced virus synthesis which, however, does

not lead to the appearance in the peripheral blood of EBNA-positive cells, at least, such cells do not become detectable by our present method. LINDAHL *et al.* [18] failed to show EBNA-positive cells in the peripheral blood of patients with chronic lymphoid leukaemia, another disease characterized by a high anti-EBV level. These authors, however, did not separate the B lymphocytes from other leucocytes.

Thus, we have failed in demonstrating EBNA-positive cells in spleen and lymph-node samples from patients with Hodgkin disease.

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EXPRESSION OF ONCORNAVIRUS ANTIGENS IN PERIPHERAL LEUCOCYTES OF PATIENTS WITH CHRONIC MYELOID LEUKAEMIA*

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Peripheral leucocytes from 16 patients with chronic myeloid leukaemia (CML) were examined for the presence of oncornavirus p30 antigens by indirect cytoplasmic immunofluorescence. The leucocytes of 12 patients who could be kept in balance by chemotherapy proved to be negative or contained the p30 antigen of mammalian endogenous oncornaviruses as the only viral antigen. In the leucocytes of four patients being in blastoid crisis, an antigen related to the p30 antigen of mammalian leukaemia-sarcoma viruses was detected. In five of six patients decrease in sensitivity to chemotherapy, or blastoid crisis, was preceded by expression of leukaemia-sarcoma virus p30 antigen(s). Leucocytes from 15 CML patients kept in balance by chemotherapy and those from seven being in blastoid crisis, were examined by indirect membrane immunofluorescence for the presence of antigen(s) related to the gp70 antigen of the simian and murine leukaemia-sarcoma virus. All tests proved to be negative.

A few years ago, nucleic acid sequences related to those of mammalian oncornavirus RNA were detected by means of the nucleic acid hybridization procedure in leucocytes of patients with various types of leukaemia [1]. When, however, type-C oncornaviruses had been isolated from patients with acute leukaemia [2, 3], the studies on the relationship between chronic leukaemias and oncornaviruses fell into the background.

Recently, we have succeeded in demonstrating antigens related to the p30 polypeptide antigen of mammalian oncornaviruses in leucocytes of patients suffering from acute or chronic myeloid leukaemia [4]. Patients in the quiescent phase of chronic myeloid leukaemia (CML) and those easily kept in balance with chemotherapy differed in the antigen spectrum of their leucocytes from patients being in blastoid crisis.

In the present work an attempt was made to confirm the relationship between antigen spectrum and clinical status in a greater number of patients on the one hand and to study the possible prognostic importance of antigen expression. The investigations have been extended to the gp70 antigen [5] as well.

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Materials and methods

Leucocytes were purified as described by BOYLE [6]. Erythrocytes were removed by lysis in 0.83% ammonium chloride solution [4].

Antisera and fluorescent conjugates. The following goat antisera were used. Anti-Rauscher leukaemia virus (RLV) p30, anti-RD-114 virus p28, anti-feline leukaemia virus (FeLV, Rickard strain) p27, anti-baboon endogenous oncornavirus (BaEV, M7 strain) p28 and anti-simian sarcoma virus (SSV) p28. All these as well as the goat antisera to the detergent-revealed gibbon leukaemia virus (GaLV and gp70 antigen of RLV) were kindly supplied by Dr. R. WILSNACK (Huntingdon Research Laboratory, Brooklandville, Md., USA). In indirect immunofluorescence (IF) studies, FITC-labelled anti-goat rabbit serum (Hyland Laboratory, Costa Mesa, Calif., USA) was used.

Indirect cytoplasmic IF. One drop of each cell sample containing $1.4\text{--}1.6 \times 10^5$ cells was placed on a slide, allowed to dry and fixed in acetone. In the first phase of the reaction one drop of anti-SSV, p28, anti-BaEV p28, anti-FeLV p27, anti-RD-114 p28 or anti-RLV p30 was added to the fixed cells. Each antiserum was used at a dilution the tenfold of its immunodiffusion titre. In the second phase, one drop of 1 : 10-diluted FITC-labelled anti-goat IgG serum was added to each preparation. Details of the procedure have been published elsewhere [4].

Indirect membrane IF. For this purpose cell suspensions of at least 80% viability were used. To keep cells viable, they were stored at 4 °C, except during the incubation periods, and the PBS of pH 7.2 used for washing cells was kept in melting ice. In the first phase of the reaction 0.05 ml antiserum was added to 2×10^6 target cells; in the second phase the cells were incubated with 0.05 ml of the tenfold diluted conjugate.

In examining cells for antigen of gp70 specificity, we used the goat anti-RLV gp70 and the anti-GaLV (detergent-treated) serum. The antisera were used in the dilution showing ring-like IF in 100% of the homologous systems. The specificity of the anti-GaLV positive reaction was proved by the GaLV gp70-IF-blocking activity of the serum. The GaLV gp70 antigen was kindly supplied by Pfizer Laboratories (Pfizer International Inc., New York, N. Y., USA).

Results

Detection of cytoplasmic (p30) antigen(s). In individual samples, either all cells were found to be negative or 80–100% of them showed a positive reaction.

Data for the 16 untreated CML patients admitted in 1977 are presented in Table I. The leucocytes of 5 of the 12 patients easily kept in balance by chemotherapy proved to be negative; in those of the remaining 7 the p30 antigen of endogenous type-C oncornavirus (RD-114, BaEV) could only be detected. The leucocytes from the 4 patients admitted in the blastoid phase of CML showed an antigen profile characteristic of AML [4], viz., each cell sample gave a positive reaction with the anti-SSV p28 serum and, in addition, the leucocytes of two patients (K. I. and T. J.) reacted with the anti-RLV p30 serum. With the anti-endogenous oncornavirus p30 sera, antigen was detected in leucocytes of three patients (S. J., K. I., and T. J.).

Table II presents the data of the CML patients who showed changes in the course of their illness during the two-year observation period. The reactions obtained at three different points of time are given for each patient. The first date corresponds to that of the first blood sampling, the second to the first change observed in the p30 antigen spectrum and the third to

Table I

Mammalian oncornavirus p30 antigens in peripheral leucocytes of patients with chronic myeloid leukaemia

Initials, sex and age of patients	Diagnosis	Leucocytes		p30 antigen spectrum				
		per μ l blood	blasts, per cent	SSV	BaEV	RD-114	FeLV	RLV
K. F. ♂ 43	CML	3 800	0	—	+	+	—	—
R. J. ♂ 45		23 700	1	—	+	+	—	—
R. M. ♀ 49		12 400	0	—	+	+	—	—
K. J. ♂ 59		36 700	1	—	+	+	—	—
K. L. ♀ 47		25 400	0	—	—	—	—	—
T. S. ♀ 53		17 100	0	—	+	—	—	—
B. I. ♀ 39		10 400	0	—	+	—	—	—
D. M. ♂ 66		24 800	2	—	—	—	—	—
R. S. ♀ 43		12 400	0	—	—	—	—	—
A. J. ♀ 55		9 300	0	—	+	—	—	—
I. J. ♀ 45		110 000	1	—	—	—	—	—
B. F. ♀ 45		50 000	1	—	—	—	—	—
S. J. ♀ 55	CML blastoid phase	86 000	5	+	+	+	—	—
K. I. ♂ 26		26 000	21	+	+	+	—	+
K. A. ♂ 59		9 400	6	+	—	—	—	—
T. J. ♂ 49		16 000	5	+	+	+	—	+

the onset of the change in clinical picture. In the first three cases (N. I., Sz. I. and K. J.) the quiescent phase of CML turned into blastoid crisis. In patients N. I. and Sz. I. the antigen spectrum changed 5 and 2 months respectively, before, in case K. J. at the time of the onset of, the blastoid crisis. The remaining 3 patients developed resistance to chemotherapy without blastoid crisis 5 months (N. S.) and 1 month (Sz. J. and K. I.) after the expression of the antigens characteristic of acute leukaemia. Keeping these cases in balance met with difficulties even after an increased dosage of cytostatics had been introduced.

Of the control subjects, 87 in number, 44 were healthy persons, the others were suffering from diseases other than leukaemia, among them 5 from cytopenia distinguishable from preleukaemia. Cytoplasmic fluorescence with antiserum to the p30 antigen of endogenous simian oncornavirus was shown in two control patients neither of whom was suffering from cytopenia.

Search for gp70 antigen on the surface of leucocytes. Table III shows the results of testing for gp70 antigen of 8 cases of AML and 10 cases of CML. In 6 of the 8 acute cases, an antigen related to GaLV gp70 was detected on

Table II

Changes in the clinical picture and in the p30 antigen spectrum of patients with chronic myeloid leukaemia

Initials, sex and age of patients	Date of blood sampling	Diagnosis	Leucocytes		p30 antigen spectrum				
			per μ l blood	blasts, per cent	SSV	BaEV	RD-114	FeLV	RLV
N. I. ♂ 55	21/5/76	CML	13 500	0	—	—	—	—	—
	18/6/76	CML	7200	0	+	+	+	—	+
	25/11/76	CML blastoid phase	29 400	43	+	+	+	—	+
Sz. I. ♀ 64	21/5/76	CML	13 300	0	—	—	—	—	—
	18/6/76	CML	10 700	0	+	+	—	—	—
	17/8/76	CML blastoid phase	12 000	13	+	+	+	—	—
K. J. ♀ 67	16/12/76	CML	25 000	0	—	+	—	—	—
	9/6/77	CML pre-blastoid phase	69 200	9	+	+	+	—	—
	14/9/77	CML blastoid phase	49 000	27	+	+	+	—	+
N. S. ♀ 52	21/5/76	CML	22 000	1	—	+	—	—	—
	9/6/77	CML	47 600	1	+	+	—	—	—
	17/11/77	CML	107 000	1	+	+	+	—	+
Sz. J. ♂ 38	7/10/76	CML	45 600	0	—	—	—	—	—
	17/11/77	CML	55 000	1	+	+	+	—	—
	1/12/77	CML	103 000	2	+	+	+	+	+
K. I. ♂ 81	27/10/77	CML	31 800	0	—	—	—	—	—
	1/12/77	CML	51 900	2	+	+	+	—	—
	12/1/78	CML	55 600	1	+	+	—	—	+

the surface of peripheral leucocytes. Those of one patient gave an additional positive reaction with the anti-RLV gp70 serum. In one patient with AML and another with myelomonocytic leukaemia (K. Gy. and V. P.) no antigen of gp70 specificity could be detected.

The first 6 patients were in the active phase of leukaemia, while the remaining two, though having high blast cell counts, soon turned into remission. Among the 10 CML patients in Table III, 7 were in the blastoid phase, 3 were kept in balance by chemotherapy with difficulty. We failed to detect any antigen of gp70 specificity in the leucocytes of these patients.

Peripheral leucocytes of the first 12 patients listed in Table I, all being in the quiescent phase of CML, were also tested for gp70 antigen, in each case with a negative result.

Gp70 antigen could not be detected on the surface of leucocytes of the 44 healthy control subjects.

Table III

Antigen(s) of gp70 specificity on the surface of leucocytes of patients with acute or chronic myeloid leukaemia

Initials, sex and age of patients	Diagnosis	Leucocytes		Results as tested with anti-	
		per μ l blood	blasts, per cent	GaLV	RLV
				serum	
P. M. ♂ 26	AEL	61 000	78	+	+
N. B. ♂ 55	AML	16 700	10	+	—
M. P. ♀ 62	AML	28 000	93	+	—
Sz. J. ♂ 40	AMML	3 000	10	+	—
K. I. ♂ 44	AMML	5 400	62	+	—
T. T. ♂ 43	AML	158 000	96	+	—
K. Gy. ♂ 43	AML	7 600	95	—	—
V. P. ♂ 65	AMML	7 400	36	—	—
S. J. ♀ 55	CML	86 000	5	—	—
K. I. ♂ 26	blastoid phase	26 000	21	—	—
K. A. ♂ 59		9 400	6	—	—
T. J. ♂ 49		16 000	5	—	—
K. J. ♀ 67		49 000	27	—	—
Sz. J. ♀ 55		14 700	25	—	—
C. Gy. ♂ 55		22 400	24	—	—
N. S. ♀ 52	CML	107 000	1	—	—
Sz. J. ♂ 38	CML	103 000	2	—	—
K. I. ♂ 81	CML	55 600	1	—	—

Discussion

The present results have confirmed our earlier observations [4] that in the quiescent phase of CML only the p30 polypeptides of endogenous (non-transforming) mammalian oncornaviruses are detectable in the cytoplasm of peripheral leucocytes. In cases showing an intensive cell proliferation, thus being kept in balance with difficulty, as well as in patients in blastoid crisis, antigens serologically cross-reacting with p30 antigen(s) of oncogenic leukaemia-sarcoma viruses are present. LARSEN *et al.* [7] succeeded in demonstrating an RNA hybridizing with the DNA copy of the Moloney leukaemia-sarcoma virus genome in leucocytes of 9 out of 15 patients with AML, but in none of 5 CML patients. AULAKH and GALLO [8], on the other hand,

succeeded in detecting a nucleotide sequence related to the DNA copy of RLV in leucocytes of one of three CML patients. These findings suggest that the mammalian leukaemia-sarcoma virus genome is present in leucocytes even in the quiescent phase, as a rule in a completely repressed state. In a minority of the cases, repression of the genome seems to be partial; in such cases its presence is detectable by the sensitive hybridization technique, but not by IF techniques. Thus, the method employed by us fails to distinguish between partial and complete repression. Presumably, the frequently observed expression of the p30 of endogenous oncornaviruses may be explained by a similar mechanism. We succeeded in demonstrating such an antigen in 50% of patients with leukaemia and in 1-2% of the control subjects.

WONG-STAAAL *et al.* [9] arrived at the same result in another way. They detected a DNA hybridizing with the RNA of the endogenous virus BaEV in leucocytes of healthy subjects and patients with myeloid leukaemia. The frequency was 23% in healthy subjects and 70% in the patients.

In 5 of 6 CML patients enhanced cell proliferation or blastoid crisis was preceded by a positive change in the p30 profile of leucocytes. The time interval ranged from 1 to 5 months. It seems that the method used by us will prove sensitive enough to be used as a prognostic tool. This view, however, needs support by a great number of similar observations.

We have reported the presence of antigen(s) related to the gp70 structural component of mammalian leukaemia viruses on the surface of peripheral leucocytes of patients suffering from acute leukaemia. The two patients turning into remission proved to be negative. In studying a great number of cases in remission we failed to find any gp70-positive cell [10]. In none of 22 patients in different phases of CML was gp70 antigen detectable on the surface of peripheral lymphocytes. METZGAR *et al.* [11], who tested with anti-Friend leukaemia virus gp70 serum the leucocytes from 21 patients of whom none was in the blastoid phase, found that all the cases were negative. We used, among other antisera, a serum to primate leukaemia virus gp70, and the tested patients included 7 in the blastoid phase. Nevertheless, we failed to detect gp70 antigen.

It may therefore be concluded that in CML blastic transformation results in the expression of p30 antigen(s), but is not accompanied by that of antigen(s) related to the gp70 glycoprotein of mammalian leukaemia virus. The lack of detectable gp70 expression might be attributed to an immunological elimination of rarely occurring gp70-positive blast cells. This assumption would be supported by the demonstration of anti-gp70 in CML sera.

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DAS ANODISCH WANDERnde THERMOLABILE ANTIGEN (ATA) VON GRAMNEGATIVEN BAKTERIEN. SCHUTZ VON MÄUSEN DURCH IMMUNISIERUNG MIT ATA

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(Eingegangen am 20. März 1978)

G. SELTMANN and R. REISSBRODT (*Institute for Experimental Epidemiology, Wernigerode, GDR*): The anodically moving thermolabile antigen (ATA) of Gram negative bacteria. Protection of mice by immunization with ATA. Mice are protected against lethal doses of *Salmonella typhi-murium* by vaccination with ATA extracted from *Providencia*. No protective effect is observed when the *S. typhi-murium* strains are enterotoxigenic. Thus protection is antibacterial, but not antitoxic.

Mäuse können durch Impfung mit aus *Providencia* extrahiertem ATA gegen letale Dosen von *Salmonella typhi-murium* geschützt werden. Es kann keine Schutzwirkung beobachtet werden, wenn die *S. typhi-murium*-Stämme enterotoxinogen sind. Der Schutz ist also antibakteriell, aber nicht antitoxisch.

Kürzlich konnten wir zeigen, daß das anodisch wandernde thermolabile Antigen von gramnegativen Bakterien (ATA) nach Extraktion aus der Bakterienzellwand sowohl in Mäusen als auch in Kaninchen die Bildung spezifischer Antikörper induziert [1].

Bisher war nicht bekannt, ob diese Antikörper protektiv sind und ob sich ein evtl. Schutz nur gegen den Serotyp des Produktionsstammes oder auch gegen Stämme anderer Serotypen oder Genera richtet.

Nach den vorliegenden Ergebnissen schützen die gebildeten Antikörper auch gegen Stämme anderer Genera, sofern diese nicht Exotoxine (z. B. Enterotoxine) produzieren.

Material and Methoden

Bakterienstämme. Die verwendeten Stämme sind in Tabelle I zusammengestellt.

Isolierung des ATA. ATA wurde nach REISSBRODT *et al.* [2] aus *Providencia* Stamm 4619 gewonnen. Das isolierte ATA wurde durch Zusatz von Gelatine stabilisiert. Reinheit und Identität des isolierten ATA wurden in der Immundiffusion gegen die monospezifischen Antiseren [1] getestet.

Herstellung des Rohenterotoxins. Das Rohenterotoxin stellt den lyophilisierten Kulturüberstand eines mit Toxinmedium [3] angezüchteten *Escherichia coli* 10407 dar. Der Proteingehalt betrug 10%, 10 mg des Präparates in 0,5 ml 0,85%igem NaCl gelöst ergaben im PF-Test eine Bläuungszone von 10 × 10 mm [4].

Immunisierung und Belastung. Die Mäuse wurden an 5 aufeinanderfolgenden Tagen i. p. mit je 0,2 mg ATA immunisiert [5]. Am 3. Tag nach der letzten Immunisierung wurden zwei Tiere zur Testung des Antikörpergehaltes getötet, die restlichen Tiere gemeinsam mit einer Kontrollgruppe mit einer *S. typhi-murium*-Dosis belastet, die in Vorversuchen innerhalb von 25 Tagen ca. 80% der Tiere tötete. Bei der Immunisierung mit dem Rohenterotoxin von *E. coli* 10407 wurden 0,2 mg Protein/Tag und Maus i. p. beigemischtem Impfschema gegeben.

Vorbereitung der *S. typhi-murium*-Stämme. Die verwendeten Stämme und ihre Charakteristik sind in Tabelle I angegeben. Sie wurden in Nährbouillon I (unter Zusatz von 20% Ascitesflüssigkeit) eingepflegt und 6 Std. bei 37 °C unter Schütteln kultiviert. Aus der Bouillon wurde auf mit 1% Ascites und 2% Fleischextrakt supplementierte Nähragar I-Platten (Nährbouillon I und Nähragar I SIFIN Berlin-Weißensee, DDR) aufgeschwemmt, dann über Nacht bei 37 °C bebrütet. Die Bakterien wurden mit 0,85%iger NaCl-Lösung abgeschwemmt, dann wurde zentrifugiert und die Bakterien 2 × mit 0,85%iger NaCl-Lösung gewaschen. Mit Hilfe eines Trübungsstandards wurde die Keimzahl bestimmt und auf die in Vorversuchen ermittelte Keimzahl zur Infektion verdünnt. Die genaue Keimzahl wurde durch Ausplattieren auf Nähragar I-Platten bestimmt.

Keimzahlbestimmung in den Organen. Am 5. Tag nach der Belastung wurden je 5 Tiere der Testgruppe und der Kontrollgruppe getötet und Leber, Milz und Niere nach Zerkleinern in jeweils 20 ml Anreicherungsmedium gegeben. Zusammensetzung des Mediums: Pepton pankreat. 20,0 g; NaCl 5,0 g; Hefeextrakt »Bramsch« 20,0 g; Rindergalle 100,0 ml; Glycerin 20,0 ml; Bromthymolblau 240 mg; Harnstoff 1,0 g; Aqua dest. 1000 ml.

Es wurde 4 Std. bei 37 °C geschüttelt, anschließend wurden 5 ml der Anreicherung zentrifugiert.

Das Sediment wurde einmal mit 0,85%iger NaCl-Lösung gewaschen, anschließend in 5,0 ml 0,85%iger NaCl-Lösung suspendiert. Nach Herstellung einer Verdünnungsreihe wurde 0,1 ml der entsprechenden Verdünnungsstufen auf Agarplatten aufgeschwemmt, die Platten wurden über Nacht bei 37 °C bebrütet und die Keimzahl wurde am nächsten Tag abgelesen.

Parallel zur Titerbestimmung erfolgte eine Reinheitskontrolle auf der Galle-Chrysoidin-Glycerin-Platte [6]. Aus allen Organen war *S. typhi-murium* in Reinkultur isoliert worden.

Bestimmung der PF-Aktivität. Der kapillare Permeabilitätstest zur Bestimmung von hitzelabilen Enterotoxinen wurde in der von TSCHÄPE und Mitarb. [4] beschriebenen Weise durchgeführt.

Tabelle I

Verwendete Salmonella typhi-murium-Stämme zur Belastung

(alle Stämme wurden der Stammsammlung des IEE Wernigerode entnommen)

Stamm	Lysotyp (Felix-Callow)	Antibiogramm	PF-Aktivität*
M668 LT2	2	Str, OTC, Neo, Cm, sensibel	—
85/77	2	Str, OTC, Cm, Kana, Amp, sensibel	(12 × 12)
234/77	1	Str, OTC, Cm, Neo, sensibel	16 × 16
575/77	n. c. 1/72	Str, Cm, Neo, sensibel OTC resistent	18 × 18 (hämorrhag.)

* PF-Aktivität = Bläunungszone (mm) einer 16 Std. Kultur des Stammes (s. Material u. Methoden).

Ergebnisse

Belastung mit *S. typhi-murium* LT2. Der Stamm zeigte keine PF-Aktivität, produzierte also offenbar kein Enterotoxin. Seine Virulenz ist ziemlich schwach, erst 10^7 Keime pro Maus erzeugten bei i. p.-Injektion eine 80–90%ige Sterberate; 10^8 Keime pro Maus töteten innerhalb von 7 Tagen alle Tiere.

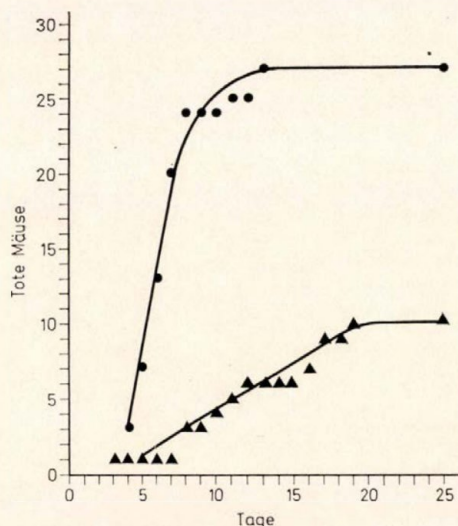


Abb. 1. Verlaufskurve der Mortalität bei Belastung mit *S. typhi-murium* LT2 ($3,5 \times 10^7$ Keime/ml). ●—● ohne Immunisierung, ▲—▲ immunisiert mit ATA

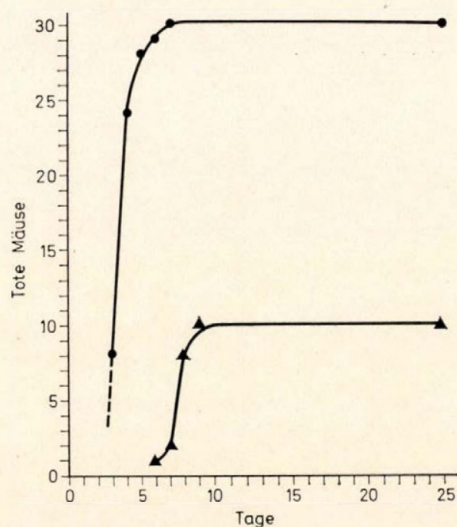


Abb. 2. Verlaufskurve der Mortalität bei Belastung mit *S. typhi-murium* LT2 ($2,5 \times 10^8$ Keime/ml). ●—● ohne Immunisierung, ▲—▲ immunisiert mit ATA

Nach Immunisierung der Tiere mit ATA war ein deutlicher Schutz gegen Belastung mit diesem Stamm feststellbar. Abb. 1 zeigt den Verlauf der Absterberate bei Belastung mit $3,5 \times 10^7$ Keimen pro Maus, Abb. 2 den bei Belastung mit $2,5 \times 10^8$ Keimen. Für die Belastungen mit 10^7 bzw. 10^8 Keimen liegt χ^2 bei 18,1 bzw. 17,5, die Irrtumswahrscheinlichkeit $<0,1\%$.

Belastung mit S. typhi-murium 85/77. Dieser Stamm zeigte geringe PF-Aktivität, der rohe Kulturüberstand der 16 Std.-Anzüchtung ergab eine nur schwache Bläuungszone mit einem Durchmesser von 12×12 mm. Gegen-

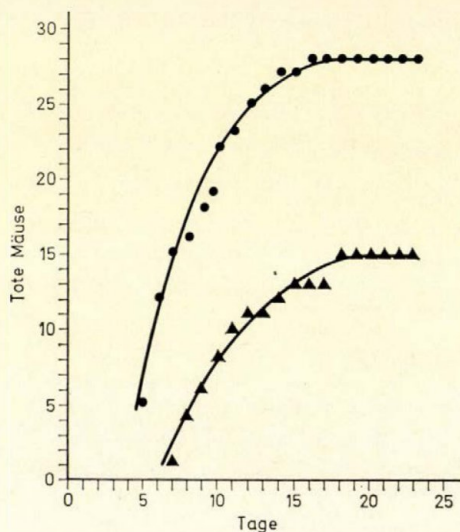


Abb. 3. Verlaufskurve der Mortalität bei Belastung mit *S. typhi-murium* 85/77 ($5,0 \times 10^5$ Keime/ml). ●—● ohne Immunisierung, ▲—▲ immunisiert mit ATA

über dem Stamm LT2 war seine Virulenz erhöht, für eine 80–90%ige Absterberate reichten 5×10^5 Keime pro Maus.

Immunisierung der Tiere mit ATA führte zu einem Schutz gegen Belastung auch mit diesem Stamm mit einer Irrtumswahrscheinlichkeit $<0,1\%$, das χ^2 lag in diesem Fall mit 11,8 aber schon deutlich schlechter als beim Stamm LT2. Abb. 3 zeigt den Verlauf der Absterberate für diesen Fall.

Belastung mit S. typhi-murium 234/77 und 575/77. Beide Stämme waren im PF-Test deutlich positiv, die Durchmesser der starken ausgeprägten Bläuungszonen lagen bei 16×16 bzw. 18×18 mm. Die Virulenz beider Stämme war mit der des Stammes 85/77 vergleichbar: für eine 80–90%ige Sterberate waren 10^5 – 10^6 Keime pro Maus notwendig.

Gegen diese Stämme war ein Schutz der Mäuse durch Immunisierung mit ATA nicht möglich (Abb. 4 u. 5).

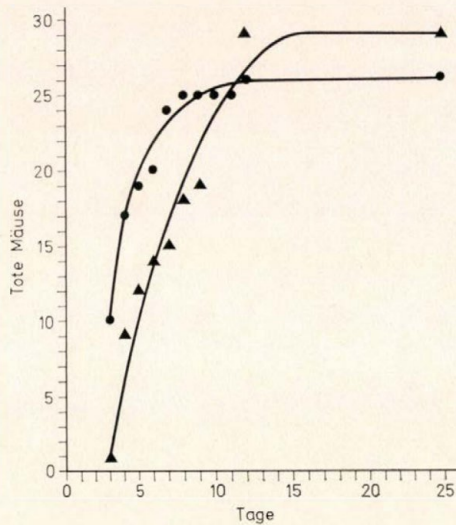


Abb. 4. Verlaufskurve der Mortalität bei Belastung mit *S. typhi-murium* 234/77 ($7,8 \times 10^5$ Keime/ml). ●—● ohne Immunisierung, ▲—▲ immunisiert mit ATA

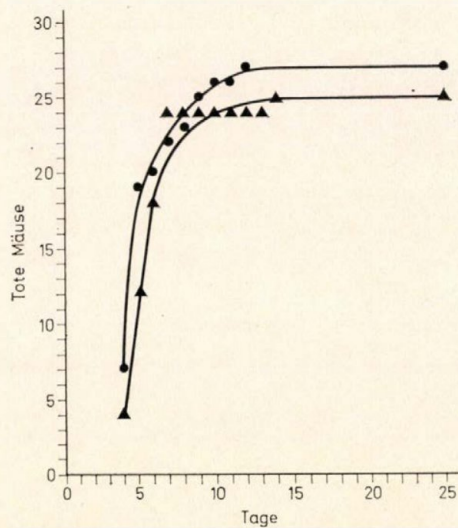


Abb. 5. Verlaufskurve der Mortalität bei Belastung mit *S. typhi-murium* 575/77 ($7,6 \times 10^4$ Keime/ml). ●—● ohne Immunisierung, ▲—▲ immunisiert mit ATA

Belastung mit Stamm 234/77 nach Immunisierung mit ATA und einem Rohenterotoxin. Die Immunisierung der Mäuse mit ATA und einem rohen Kulturüberstand des Stammes *E. coli* 10407 ergab den in Abb. 6 angegebenen Verlauf der Absterberate. Verglichen mit dem in Abb. 4 angeführten Verlauf ist ein gewisser Schutz der doppelt immunisierten Tiere nicht zu übersehen,

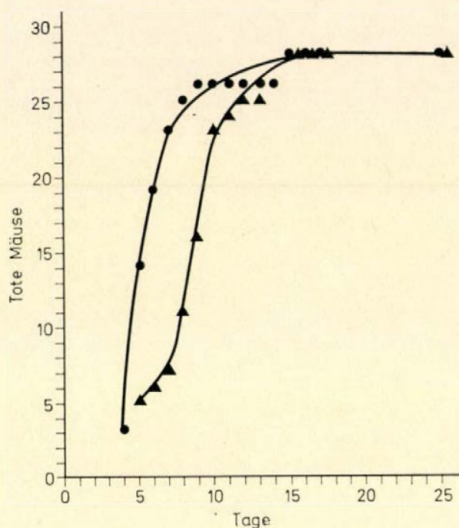


Abb. 6. Verlaufskurve der Mortalität bei Belastung mit *S. typhi-murium* 234/77 ($9,1 \times 10^5$ Keime/ml). ●—● ohne Immunisierung, ▲—▲ immunisiert mit ATA und Rohenterotoxin von *E. coli* 10407

der sich aber im wesentlichen auf eine Verlängerung der Überlebenszeit der Tiere beschränkte.

Eliminierung der S. typhi-murium-LT2-Keime aus den Organen der Tiere. Als Kontrollorgane wurden Leber, Niere und Milz von je 5 Mäusen verwendet. Tabelle II zeigt, daß nach 5 Tagen in diesen Organen bei den mit ATA immunisierten Tieren ein Rückgang des Bakterientiters auf $<10\%$ des Titers in den Organen der nicht immunisierten Tiere zu beobachten war. Dies läßt auf eine rasche Eliminierung der Bakterien bei den geimpften Tieren schließen.

Tabelle II

Keimzahlen von *S. typhi-murium* LT2 in Leber, Niere und Milz mit und ohne Immunisierung 5 Tage nach Infektion

	Durchschnittliche Keimzahlen in		
	Leber	Niere	Milz
Immunisiert	$3,9 \cdot 10^{10}$	$4,4 \cdot 10^{10}$	$4,0 \cdot 10^{10}$
Nicht immunisiert	$1,2 \cdot 10^{12}$	$6,9 \cdot 10^{11}$	$8,8 \cdot 10^{11}$

Diskussion

Immunisierung von Mäusen mit gereinigtem ATA führt bei dem *S. typhi-murium*-Stamm LT2 zu einem Schutz der Tiere, der weit innerhalb des Signifikanzbereiches liegt ($\chi^2 \sim 18$; Irrtumswahrscheinlichkeit $< 0,1\%$).

Dieser Stamm ist in dem als Kriterium für eine Enterotoxinproduktion angesehenen PF-Test [4, 7] negativ. Bei Stamm 85/77, der eine schwache PF-Aktivität zeigt, ist der durch Immunisierung erzielte Schutz schon etwas schwächer ($\chi^2 \sim 11$; die Irrtumswahrscheinlichkeit liegt aber immer noch $< 0,1\%$).

Anders liegen die Verhältnisse bei den *S. typhi-murium*-Stämmen 234/77 und 575/77. Diese Stämme zeigen eine starke PF-Aktivität. Bei ihnen ist durch Immunisierung mit ATA kein Schutz, sondern sogar eine gewisse Sensibilisierung zu beobachten. Ein teilweiser Schutz konnte aber erzielt werden, wenn die Stämme zusätzlich zum ATA mit einem rohen Kulturüberstand von *E. coli* 10407, der als starker Enterotoxinbildner bekannt ist, immunisiert wurden.

Wir können die Unterschiede in der Schutzwirkung des ATA nicht auf die unterschiedlichen Lysotypen zurückführen, die verwendet wurden. Obgleich gegen beide Stämme von Lysotyp 2 eine Schutzwirkung vorhanden ist, sind die zur Infektion und einer Tötungsrate von 80–90% nötigen Dosen doch um 3 Zehnerstufen verschieden. Dagegen sind etwa gleiche Infektionsdosen wie beim Stamm 85/77 (Lysotyp 2) bei den Stämmen 234/77 und 575/77 (Lysotyp 1 bzw. n. c. 1/72) nötig.

Unsere Ergebnisse deuten darauf, daß der durch Immunisierung mit ATA erzielte Schutz antibakteriell, nicht aber antitoxisch wirkt. Die praktische Nutzung der Ergebnisse etwa im Sinne einer Impfstoffentwicklung ist wegen der starken Enterotoxigenität der meisten *S. typhi-murium*-Stämme im Augenblick noch nicht gegeben. Weitere Untersuchungen mit gereinigten Enterotoxinpräparaten sind in Vorbereitung.

Die Schutzwirkung des ATA auf die Infektion mit *S. typhi-murium* LT2 wird durch Reduktion des Bakterientiters in den Organen von immunisierten gegenüber nicht immunisierten Tieren unterstrichen (Tab. II), eine Methode, die nach COLLINS [8] als sicherste zur Feststellung einer protektiven Wirkung von Antikörper angesehen wird.

ATA ist nicht das einzige Beispiel, daß Substanzen der Zellwand, die in den Wildstämmen nach außen hin nicht in Erscheinung treten, protektive Antikörper zu erzeugen vermögen. Die in den Wildstämmen der *Enterobacteriaceae* ebenfalls nach außen hin nicht in Erscheinung tretende Lipid A-Komponente der O-antigenen Lipopolysaccharide erzeugt in Mäusen einen 50–60%igen Schutz gegen eine Infektion mit *S. typhi-murium* [9].

Im Jahre 1966 haben COLLINS und MILNE [10] mit einem Antigen, das nach dem Präparationsschema mit dem ATA identisch sein könnte (wenn auch die deutlich geringere Thermostabilität des ATA dagegen spricht), und im Jahre 1975 KALJSER [11] mit von ihm als HMA (high mobility antigen) bezeichneten ATA ähnliche Immunisierung vorgenommen. Sie fanden keinen Schutzeffekt. In beiden Fällen findet man keinen Hinweis auf ein evtl. Toxinproduktionsvermögen der untersuchten Stämme.

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ADAPTATION OF THE INFLUENZA NEURAMINIDASE AND NEURAMINIDASE-INHIBITION ASSAYS TO THE MICROTITRATOR SYSTEM

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A micro-method based on WARREN's colour reaction has been developed for influenza neuraminidase and neuraminidase-inhibition assays. Extraction with butanol is omitted and the assay is performed in wells of special plexiglass trays, where the reaction mixture is electrically heated to reading-temperature. The result is read by visual inspection and compared to controls in the same tray. The micro-assay, being inexpensive, time-saving and easy to perform even in poorly equipped laboratories, is suitable for large-scale serological screening and identification of the neuraminidase antigen of isolates.

Among the antibodies to the influenza virus surface antigens, anti-haemagglutinin can be assayed by simple tests such as the haemagglutination-inhibition (HI) test, the complement-fixation test, the HI test combined with antibody absorption [1, 2] and various immunodiffusion methods [3, 4]. Anti-neuraminidase (anti-NA) is much more difficult to examine in large-scale work. Literary data in general rely upon WARREN's [5] colorimetric reaction, which is rather complicated and laborious even in its modified [6–9] and standardized [10] forms, thus being less applicable for mass investigations. Only the single-radial-diffusion test published by SCHILD *et al.* [3, 4] is promising in this respect.

The advantages and the widespread use of the Microtitrator system [11] has prompted us to adapt the complicated method of WARREN to this micro-system and to develop a simple test for large-scale investigations.

Reagents and instruments

The reagents described by HENRY-AYMARD *et al.* [10] are used, except the periodate reagent, which is diluted with an equal volume of distilled water.

The instruments are available from Labor MIM, 1450 Budapest, Hungary. Patent pending.

(1) "N" trays. These are plastic trays, 130 × 90 × 15 mm in size, with 6 × 10 wells 13 mm in depth (Fig. 1). The upper 10-mm part of the well is cylindrical, 8 mm in diameter, its lower part is identical in shape and size to the Microtitrator (or Microtiter) "V" wells. The total volume is approximately 0.6 ml. The tray is provided with boreholes for fitting electric heaters.

(2) Electric heaters. These serve for direct heating of reaction mixtures in the wells. Each heats 2 rows, i.e. 20 reaction mixtures, by special electric heater projections. Three heaters are used for each tray; they are mounted on the "N" tray by small metal rods, fitted into boreholes of appropriate calibre on the edges of the tray.

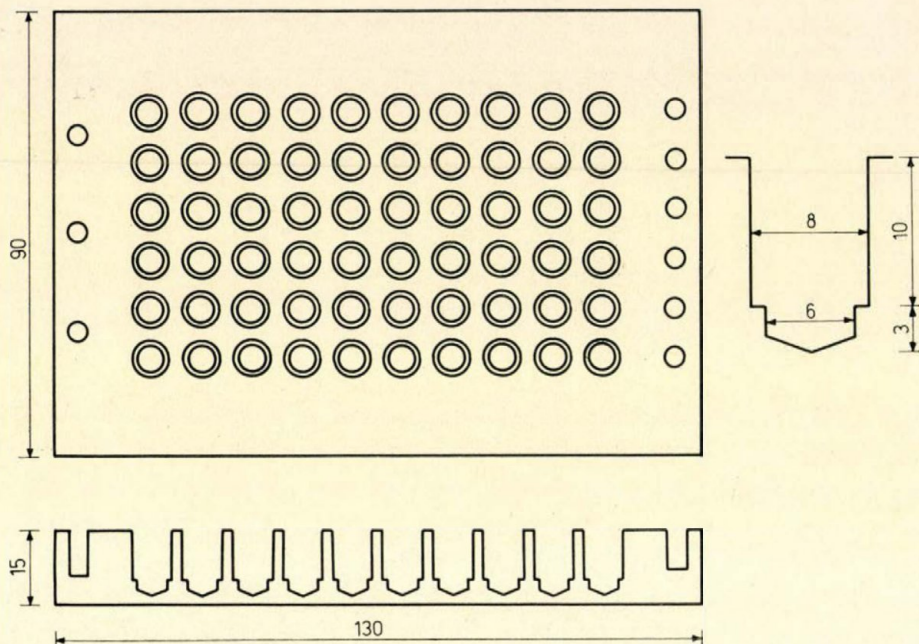


Fig. 1. The "N" tray

(3) *Electric feed-transformer unit.* This supplies stabilized low voltage (adjustable between 5 V and 22 V) for heaters (Picture 1, colour plate).

(4) *Microdiluters and droppers.* These are the same as those applied in the Microtitrator system in general.

Assays

Determination of the concentration of substrate and N-acetyl-neuraminic acid (NANA) standard. Adjusting of the enzyme activity of different virus preparations to an equal level is a prerequisite of comparable results. The enzyme activity splitting 50% of the bound NANA present in the substrate under given experimental conditions may be used favourably as an arbitrary unit. The substrate concentration should be high enough to yield a well-measurable colour intensity. With the standard technique [10] this concentration, corresponding to $OD = 0.85$, represents $125 \mu\text{g}/\text{tube}$ of NANA; in the microtechnique one-tenth of this, i.e. $12.5 \mu\text{g}/\text{well}$ of NANA, is sufficient.

Preparation of standard substrate and NANA solutions (Fig. 2). Prepare from the substrate (10% NANA content) a dilution containing 20 mg protein/ml in 0.2 M sulphuric acid and hydrolyse by boiling for 20 min.

1. Drop $50 \mu\text{l}$ 0.15 M saline in two rows of a tray.
2. Prepare two parallel series of twofold dilutions of the hydrolysate in wells No. 1-9, using the $50\text{-}\mu\text{l}$ microdiluter. Add $50 \mu\text{l}$ substrate solution

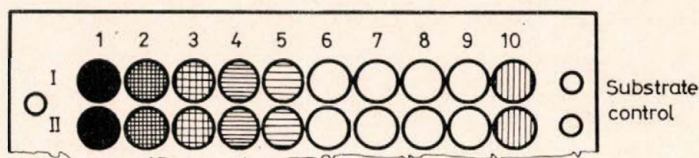


Fig. 2. Scheme for determination of dilution of substrate and NANA standards. Steps of performance: 1. 50 μ l saline I-II/1-10; 2. 50 μ l hydrolysate of substrate (I) I-II/1-9 serial twofold dilutions, I-II/10 50 μ l substrate; 3. 50 μ l saline in each well; 4. 25 μ l periodate reagent. Incubation at 25 $^{\circ}$ C for 15 min; 5. 50 μ l arsenite reagent. Incubation at 25 $^{\circ}$ C for 1 min; 6. 250 μ l thiobarbituric acid reagent. Incubation at 90–100 $^{\circ}$ C for 7 min. The dilution of sample: No. 2. Dilution of substrate for reaction. No. 3. Dilution of NANA for neuraminidase assay. No. 4. Dilution of NANA for inhibition test

to well No. 10 by a 50- μ l microdiluter. These controls serve for the detection of free NANA.

3. Add 50 μ l saline to each dilution.

4. Drop 25 μ l periodate solution into each well, mix thoroughly, allow to stand for 20 min at 20 $^{\circ}$ C or for 15–19 min at an air temperature between 21 and 25 $^{\circ}$ C. (Air temperature in $^{\circ}$ C plus minutes of incubation should equal 40.)

5. Add 50 μ l arsenite reagent to each mixture and shake by tapping the tray until the brown colour disappears (about 1 min).

6. Add 0.25 ml thiobarbituric acid reagent to each mixture by a suitable dispenser. Mount the heater on the tray and adjust the knob of the electric feed-transformer unit to mark 15 on the scale. Switch on, and heat for exactly 75 sec, then adjust the knob to mark 4 and keep so for 6 min. The desired 90–100 $^{\circ}$ C temperature is reached in 2–3 min and remains at the same level until the end of the heating period.

7. Switch off the feed-transformer unit, remove the heaters and read the reaction immediately.

The colour intensity of the wells reflects the different NANA contents of the hydrolysate dilutions; namely, the second well corresponds to the colour intensity of NANA originating from 5 mg/ml substrate, which represents the substrate dilution to be used in the test; the third corresponds to the hydrolysate dilution to be used for neuraminidase (NA) assay, and the fourth to be used as a standard solution for the neuraminidase-inhibition (NI) reaction. Well No. 10 should not show any pink colour, i.e. no visible free NANA present in the substrate. (Picture 2A, colour plate.)

If the bound NANA content of the substrate is unknown, the dilution to be used of both substrate and standard can be satisfactorily estimated as follows. The reaction is performed in serial twofold dilutions of a substrate hydrolysate of known concentration (between 20 and 50 mg/ml). The wells are then numbered from left to right. If, e.g., No. 6 is the highest dilution

developing colour, the substrate dilution in well No. 2 is to be chosen as a working-dilution of the substrate, the NANA dilution in well No. 3 as standard dilution of NANA for the NA assay, and that in well No. 4 as a standard dilution of NANA for the NI assay.

Assay of NA activity (Fig. 3)

1. Drop 25 μ l 0.15 M saline into each well of two rows of a tray.
2. Make serial twofold dilutions of virus with microdiluters in wells No. 1–9. Well No. 10 in row I serves as the standard NANA control, and that in row II as blank control.

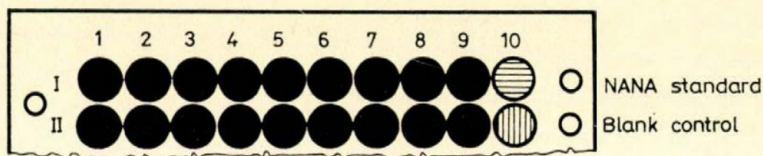


Fig. 3. Scheme of neuraminidase assay. Steps: 1. 25 μ l saline I–II/1–10; 2. 25 μ l virus (1), I–II/1–9 serial twofold dilutions; 3. 25 μ l saline in each well; 4. 50 μ l substrate I/1–9; II/1–10; 50 μ l NANA standard I/10. Incubation at 37 °C for 18 hr. 5, 6, 7: see steps 4, 5, 6 of Fig. 2

3. Drop 25 μ l saline into each well.
4. Add to wells No. 1–9 in row I and to wells No. 1–10 in row II 50 μ l substrate solution diluted in phosphate-buffered saline pH 5.9, containing 6 mM CaCl_2 . Drop the same volume of standard NANA into well No. 10 of row I. Mix well the reaction mixtures by tapping the tray. Cover the tray with another tray and allow to stand at 37 °C for 18 hr.
5. Cool the trays to room temperature and add reagents as in steps 4 through 6 of the preparation of standard NANA solutions.

The virus dilution showing the same colour intensity as standard NANA (well No. 10 in row I) is regarded as NA titre. Blank control (well No. 10 in row II) should not show any pink colour, i.e. absence of free NANA (see the rows 1 and 2 in Picture 2B, colour plate).

NI assay (Fig. 4)

1. Drop 25 μ l 0.15 M saline into each well of two rows of a tray.
2. Make serial twofold dilutions of sera with 25 μ l microdiluters in wells No. 1–9 in rows I and II.
3. Drop into wells No. 1–9 of both rows 25 μ l of the virus dilution corresponding to the NA titre. Mix the reaction mixtures, cover the tray with another tray and incubate at 37 °C for 1 hr.

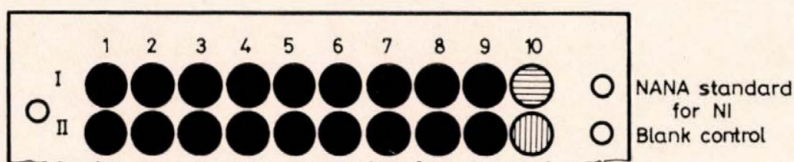
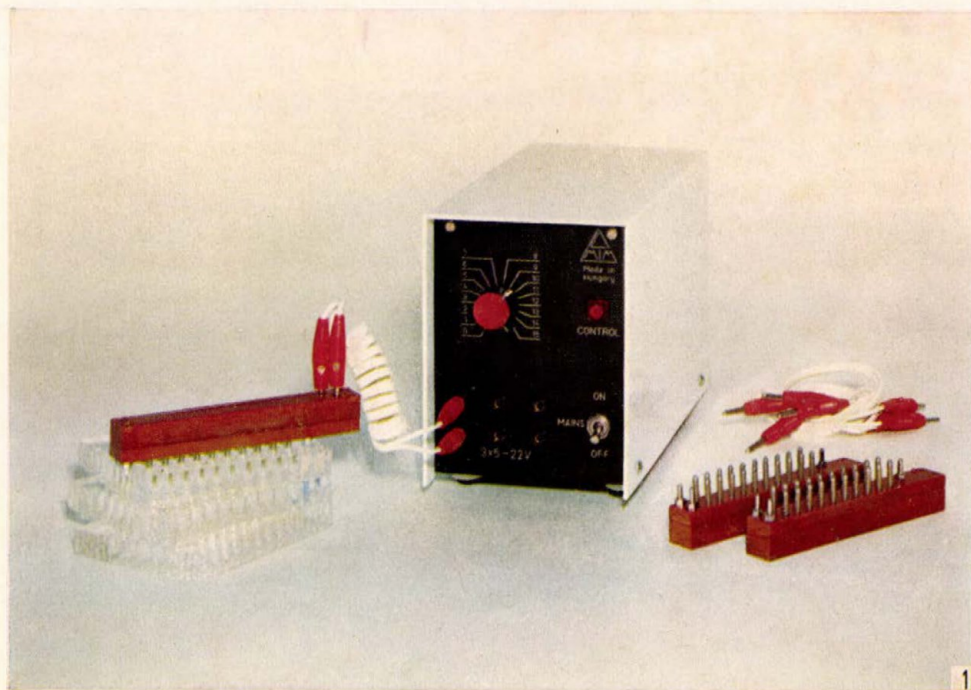


Fig. 4. Scheme of neuraminidase inhibition test. Steps of performance: 1. 25 μ l saline I-II/1-10; 2. 25 μ l antiserum (2), I-II/1-9 serial twofold dilutions; 3. 25 μ l virus dilution I-II/1-9. Incubation at 37 °C for 1 hr; 4. 50 μ l substrate I/1-9, II/1-10; 50 μ l NANA standard for NI I/10; 25 μ l saline I-II/10. Incubation at 37 °C for 18 hr. 5-7: see steps 4-6 of Fig. 2

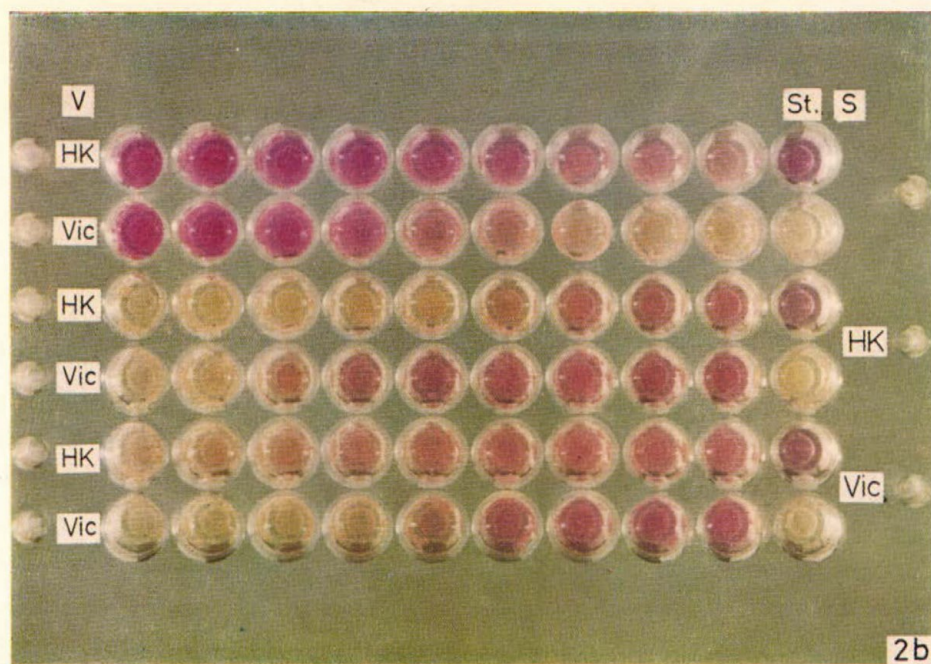
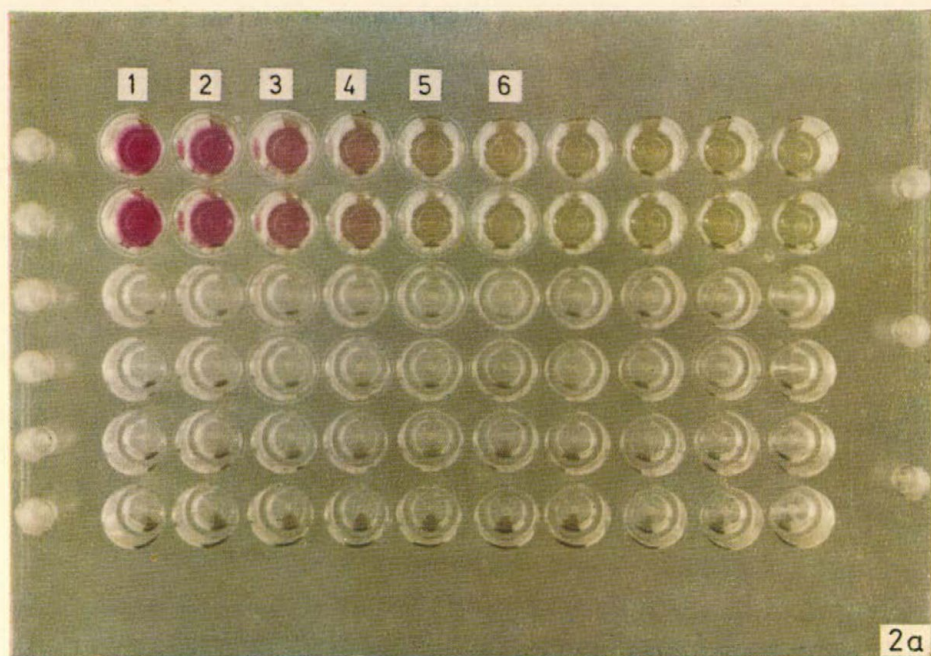


Picture 1. Electric feed-transformer unit and electric heaters

4. Add to wells No. 1-9 in row I and to No. 1-10 in row II 50 μ l substrate and 50 μ l NANA standard solution (corresponding to 50% inhibition) to the reaction mixture in well No. 10 in row I and 25 μ l saline in well No. 10 in rows I and II. Mix thoroughly the reaction mixtures, cover the tray and incubate at 37 °C for 18 hr.

5. Cool the trays to room temperature and add reagents as in steps 4-7 of the preparation of standard NANA solutions.

The NI titre of a serum is represented by the dilution giving a colour intensity corresponding to the colour of the 50% inhibition standard (see rows 3 and 4 in Picture 2B, colour plate).



Picture 2A. Colour intensities of dilutions of substrate-hydrolysate

Picture 2B. A tray with neuraminidase and antineuraminidase tests. Rows 1–2: titration of enzyme activity of Hong Kong/1/68 and Victoria/3/75 virus strains. Rows 3–6: cross NI test between Hong Kong/1/68 and Victoria/3/75 strains. Well No. 10 in row I shows the colour intensity of NANA standard for NA assay; wells No. 10 in rows 3 and 5 correspond to the colour of NANA standards for 50% inhibition. Wells No. 10 in rows 2, 4 and 6 are blank controls

Discussion

The micro-assay described in this paper has the following advantages.

(i) Butanol extraction, a laborious procedure in the original assay, is omitted; (ii) the omission of spectrophotometry makes the micro-assay accessible for poorly equipped laboratories; (iii) owing to the use of the Micro-titrator system, consumption of the expensive substrate and of other reagents is reduced to one-tenth; (iv) serial dilution of six or even more samples can be performed simultaneously; (v) the reaction mixtures need no transfer in the course of the test; (vi) in trays the results are easy to survey and compare; (vii) the colour reaction re-appears at the reading-temperature even after the tray has been kept at 4 °C for several days; (viii) owing to their low pH, the standard solutions are stable at 4 °C for several months without the addition of any preservative.

The possible presence of free NANA in undiluted virus and/or of neuraminidase in undiluted serum does not disturb reading in the range of titre end points. Consequently, the respective controls may be omitted.

In addition to these advantages, the reproducibility of the titres deserves mentioning; standard deviation was found to be lower than usual for HI titres. The presence of standard colour in each tray contributes to the accuracy of the micro-assay. The titres correlate well with those of the standard assay [10] performed simultaneously.

The practicability of the micro-assay in mass screenings for anti-NA and in identification of NA derives from its two main advantages, viz. (a) microtitration of 60 serum samples requires a manipulation time (incubation times subtracted) of one hour in contrast to the 30 hr needed by the earlier assays; (b) owing to saving reagents and equipment, expenses per assay are reduced to one-tenth.

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INCIDENCE OF NON-CHOLERA VIBRIOS IN HUNGARY

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In the period 1971 to 1976, 200 non-cholera vibrio (NCV) strains were isolated in Hungary; 18 of the cultures were derived from 34 729 faecal and 182 from 237 surface water samples. Ninety-two strains belonged to the Heiberg-Smith group I and 108 to group II. Two strains failed to give the string test and 3 were pteridine resistant. The strains were classified into 48 serotypes according to SMITH's system. Faecal NCV strains belonged to serotypes 46 and 328; these serotypes did not occur in water. Of the 18 faecal strains 13 were isolated from 18 048 persons who had travelled in cholera-infected areas, and 5 strains from persons who had never left Hungary (2 from 4559 patients with diarrhoea and 3 from 6061 healthy individuals). These data indicate that although NCV are present in the environment, they play an insignificant role in enteric infections in Hungary.

Outbreaks of cholera in neighbouring countries in 1970 called for a review of diagnostic laboratory procedures, epidemiologic measures and immunization methods employed with cholera in Hungary. Measures adopted included supplying to public health laboratories *Vibrio cholerae* O polyvalent diagnostic serum and TCBS medium along with the most recent guidelines on cholera [1-12]. A screening procedure for *V. cholerae* and related organisms became compulsory for Hungarian citizens returning from cholera-infected areas. Travellers from whom cholera and/or non-cholera vibrios (NCV) were recovered were placed in isolation, treated for three days with antibiotics and discharged if the stool culture was negative three days after stopping antibiotic administration [13]. This was done to prevent the spread of potential pathogens among the population. If NCVs were derived from individuals who had not travelled in cholera areas, no isolation measures were initiated, in view of the presumed saprophytic nature of these organisms as well as of those isolated from surface water samples [14-16].

To study whether the contradicting measures were justified, further investigations were performed. In the present paper an account is given on examinations on the occurrence of NCV in Hungary. Experience gained in five years were taken into consideration for drawing practical conclusions.

Materials and methods

Bacterial strains. *Vibrio* cultures were sent to our Department for control identification by public health laboratories who had isolated them from stools of travellers returning from infected areas. *Vibrio* strains from persons with and without diarrhoea who had not visited cholera-infected areas were also studied. In addition, *vibrio* isolates from surface waters which enter Hungary from neighbouring countries were chosen at random by 8 public health laboratories. The laboratory of Somogy county supplied water samples taken at the south shores of Lake Balaton. The laboratories that participated extended their investigations of stool and water samples to include *Aeromonas* and *Plesiomonas*.

Organisms were isolated from one litre water samples adjusted to pH 9.0–9.2 with NaOH, incubated overnight and passed through a membrane filter. The membrane was immersed in 10 ml 1% peptone water and incubated at 37 °C for 4 hr, then streaked on DC and TCBS and, after incubation, isolated colonies were tested.

Identification tests. Motility was examined under a phase contrast microscope or at dark field illumination and the culture was seeded into U-tubes and read after 24 hr. Besides, tests prescribed by the WHO and recommended for the differential diagnostics of *Vibrio*, *Aeromonas*, *Plesiomonas* and *Pseudomonas* [17, 18] were carried out similarly from an 18–24 hr slant agar culture. The String test was considered positive if the suspension prepared with the reagent was no longer opaque, formed a mucus-like "string" within 60 seconds, extending to the loop, as it was lifted away from the suspension. If a drop of the suspension prepared from the reagent remained homogeneous for more than a minute, it was regarded as negative. Oxidase reaction was carried out by the standard method [19]. Møller's tests (lysine, ornithine decarboxylase, arginine dihydrolase) were read on the 5th day, indole test and nitrate reduction on the first day. F/O glucose test was prepared in HUGH and LEIFSON agar [20]. Gas from glucose and acid from D-mannose, L-arabinose, sucrose, m-inositol were tested in the presence of 1% Andrade indicator in peptone water for 5 days. Gelatin liquefaction and proteolysis were observed for 5 days. Sensitivity of the strains to pteridine and novobiocin was investigated on Mueller–Hinton-agar as modified by KONKOLY THEGE and LÁNYI [21] by means of "Resistest" discs (Institute for Serobacteriological Production and Research Human, Budapest).

Serological testing. Only those organisms which were characterized as vibrios were tested serologically.

Cultures were initially tested with cholera O serum. Living and boiled cultures were exposed to pooled cholera O and R sera using the slide agglutination method.

Vibrio cultures that failed to agglutinate in the cholera sera, were sent to the *Vibrio* Reference Laboratory (Philadelphia, Pa., USA). Slide agglutination tests with sera prepared in rabbits were conducted employing living suspensions [22].

Enterotoxin production and detection. The following strains were used to examine toxin production (designation, organism, source, who isolated the strain).

81938, NCV, stool: enteritis, MIHÁLYFI, I. (Budapest; 69521, NCV, stool: enteritis, KOLTA, F. (Tatabánya); 38246, NCV, stool: healthy, KUBINYI, J. (Budapest); 53371, NCV, stool: healthy, KISS, P. (Hódmezővásárhely); 72473, NCV, water: Balaton, BARON, F. (Kaposvár); 57119, NCV, water: Hajdúszoboszló, LAKATOS, M. (Debrecen); 39802, NCV, water: Szamos, BODNÁR, L. (Nyíregyháza); 39803, NCV, water: Kraszna, BODNÁR, L. (Nyíregyháza); 43999, NCV, water: Tisza, HEGEDŰS, M. (Szeged); 44000, NCV, water: Tisza, HEGEDŰS, M. (Szeged). Strain *E. coli* P99 (O141:K85ab, K88ab) was kindly supplied by Dr. H. W. SMITH (Houghton Poultry Research Station, Huntington, England).

Intestinal loops of adult rabbits were used to detect the ability of cultures and culture filtrates to cause accumulation of fluid. Rabbits weighing 1.8 to 2.6 kg were fasted for 48 hr prior to operation. The animals were anaesthetized with a combination of chlorpromazine hydrochloride (5 mg/kg) and thialbarbital (50 mg/kg). The jejunum was exposed through a midline incision and usually 9 test segments, 9 cm long separated by 4 cm interloops, were prepared with a single ligature of surgical silk between the segments. The first ligature was applied 60 cm below the pylorus. One–two ml samples were injected into the test segments. A positive control LT preparation from strain P99 was inoculated into the first and last segment. After injecting the test material into the loops, the abdomen was closed. Approximately 20 hr later the rabbits were killed with an overdose of thialbarbital and the result was read. The amount of fluid accumulated in the segments was expressed as the index, by dividing the weight of fluid with the weight of the gut segment. An index value of 1.0 or higher indicated a positive reaction.

When living organisms were introduced into the intestinal loops, the culture was inoculated on a blood agar plate and incubated at 37 °C for 18 hr. From this, a tube of broth

was inoculated, incubated at 37 °C for 18 hr and 2 ml of the culture (about 10^8 organisms/ml) was injected.

Filtrates of cultures to be tested for enterotoxin were prepared according to the method of GYLES and BARNUM [23] as modified by PESTI and SEMJÉN [24]. Briefly, this consisted of incubation at 37 °C for 18 hr of the culture on a blood agar plate, inoculation into tryptose broth and, after 18 hr at 37 °C, 3 ml was inoculated on the surface of tryptose agar. The growth was removed after 18 hr at 37 °C, suspended in 13 ml distilled water, sonicated for 30 min (MSE 500 W full energy setting), centrifuged at 20 000 g at 4 °C and the supernatant was passed through a 45 µm millipore filter. After checking its sterility, 2 ml of the material was injected into the ligated intestinal loop.

Results

Table I presents the regional distribution of the two hundred NCV strains isolated in the period 1971 to 1976. All in all, 18 isolations were made from faeces and 182 from water samples.

The characteristics of the 200 isolates are given in Table II. There was good agreement in most of the results, the most notable exception being in the action of the strains on mannose. Taking into account a classification scheme based upon the action of vibrio on sucrose, arabinose and mannose as proposed by HEIBERG [25] and SMITH and GOODNER [22], eight combinations are possible.

Table I

Regional distribution of 200 non-cholera vibrio strains isolated from faeces and various water samples (surface, swimming-pool water, sewage)

Public health laboratory	No. of strains	
	from faeces	from water samples
Borsod-Abaúj-Zemplén	—	9
Csongrád	2	10
Fejér	—	29
Győr-Sopron	—	19
Hajdú-Bihar	—	39
Komárom	2	18
Pest (N.I.H.)*	—	17
Somogy	—	18
Szabolcs-Szatmár	—	14
Vas	—	7
Budapest	14	2
Total	18	182

* National Institute of Hygiene, Budapest.

Table II
Characteristics of 200 non-cholera vibrio strains

Characteristics	Positive	Negative
Oxidase test	200	0
String test	198	2
Motility	200	0
Lysine decarboxylase	200	0
Arginine dihydrolase	0	200
Ornithine decarboxylase	200	0
Gelatin liquefaction	200	0
Nitrate reduction	200	0
Indole	200	0
Acid from glucose (F)	200	0
Gas from glucose	0	200
D-mannose	92	108
L-arabinose	0	200
Sucrose	200	0
m-Inositol	0	200
Inhibition by pteridine	197	3
Inhibition by novobiocin	170*	0

* Only 17 strains were examined.

Table III
Classification of 200 non-cholera vibrio strains according to the Heiberg-Smith's grouping method

Mannose	Sucrose	Arabinose	Heiberg-Smith's group	No. of strains
+	+	—	I	92
—	+	—	II	108
+	+	+	III	0
—	+	+	IV	0
+	—	—	V	0
—	—	—	VI	0
+	—	+	VII	0
—	—	+	VIII	0

As seen in Table III, 92 strains were Heiberg I and 108 were Heiberg II. No other fermentation group was found in these isolates.

Table IV presents the further differentiation of the NCV isolated from human faeces by serotyping. With the sera employed, 13 of 18 strains were

typed and Heiberg I cultures were subdivided into at least 3 different types, while Heiberg II organisms into 7 serotypes. The 13 strains from subjects who had visited cholera areas were isolated from 8 individuals. The five isolates from non-travellers originated from 5 individuals. Only one individual who had returned from Algiers had intestinal symptoms.

Table IV

Distribution by Heiberg fermentation and serotyping of non-cholera vibrio strains isolated from human faeces

Heiberg group	Serotype	No. of strains isolated from persons		Total
		never in endemic or epidemic area	returned from endemic or epidemic area	
II	11	1		1
II	12		3	3
II	14	1		1
II	27		3	3
II	29		1	1
II	46		2	2
I	50	1		1
I	328	1		1
I	non-typable		2	2
II	non-typable	1	2	3
Total		5	13	18

Of 10 620 stool samples examined by the Public Health Stations 4559 originated from diarrhoeic patients and 6061 samples from healthy persons. From the patients 2 NCV, and from healthy persons 3 NCV strains and 1 *Aeromonas* were isolated. One patient with symptoms of acute gastroenteritis harboured only NCV but no pathogenic intestinal bacteria. During an epidemic of dysentery was the other positive case detected; from this patient NCV and *Shigella flexneri* were isolated. The pathogenic role of *Aeromonas* could not be confirmed. In 1976, 8 Public Health Stations cultured 237 surface water samples at our request. NCV was isolated from 108 and *Aeromonas* from 32 samples, i.e. 45.5% and 13.5%, respectively. These data are summarized in Table VI. Two Public Health Stations, namely Fejér county and Hajdú-Bihar county are not represented in Table VI, because the former started sampling at Lake Velence in 1974, and the latter investigated all surface waters (sewage, swimming pools) that belong to the county authority. Both laboratories sent part of the NCV strains isolated to our Department

Table V

Incidence of non-cholera vibrio species (NCV) and of Aeromonas (A) in the stools of diarrhoeic patients and healthy individuals

Public health laboratory	Total No. of samples	Diarrhoeic patients			Healthy individuals		
		No. of samples	positive		No. of samples	positive	
			NCV	A		NCV	A
Baranya	200	—	—	—	200	—	—
Bács-Kiskun	271	—	—	—	271	—	—
Békés	189	189	—	—	—	—	—
Borsod-Abaúj-Zemplén	200	—	—	—	200	—	—
Csongrád	251	—	—	—	251	1	—
Fejér	1 131	876	—	—	255	—	—
Győr-Sopron	200	—	—	—	200	—	—
Hajdú-Bihar	1 184	438	—	—	746	—	—
Heves	200	—	—	—	200	—	—
Komárom	726	380	2	—	346	—	1
Nógrád	200	—	—	—	200	—	—
Pest (N.I.H.)*	1 232	907	—	1	325	—	—
Somogy	410	200	—	—	210	—	—
Szabolcs-Szatmár	300	—	—	—	300	—	—
Szolnok	246	—	—	—	246	—	—
Tolna	200	—	—	—	200	—	—
Vas	200	—	—	—	200	—	—
Veszprém	200	—	—	—	200	—	—
Zala	203	—	—	—	203	—	—
Budapest	2 877	1569	—	—	1308	2	—
Total	10 620	4559	2	1	6061	3	1

* National Institute of Hygiene, Budapest.

for identification, however, the total number of the water samples of which the identifiable NCV and *Aeromonas* strains were cultured was unspecified, thus had to be omitted.

In Table VII the serotypes of all the 182 NCV strains isolated from water samples between 1971–1976 and classified into 48 serotypes are presented. Twenty-one types (12, 13, 17, 18, 29, 31, 33, 39, 40, 50, 60, 62, 75, 107, 113, 175, 312, 323, 332, 333, 343) were isolated from water samples. Non-typable strains were found in the water samples by 10 Public Health Stations. The NCV that belonged to other serotypes were isolated from water samples. NCV isolated from stools belonged to serotypes 46 and 328; they

Table VI

Regional distribution of non-cholera vibrio (NCV) and Aeromonas (A) strains isolated from surface water samples

Public health laboratory	No. of samples	No. of strains	
		NCV	A
Borsod-Abaúj-Zemplén	30	9	—
Csongrád	27	10	—
Győr-Sopron	30	19	4
Komárom	30	16	9
Pest (N.I.H.)*	29	15	11
Somogy	30	18	—
Szabolcs-Szatmár	31	14	—
Vas	30	7	8
Total	237	108	32
Per cent		45.5	13.5

* National Institute of Hygiene, Budapest.

could not be detected in water samples. Sixteen serotypes (33, 43, 50, 56, 62, 75, 113, 115, 201, 309, 313, 323, 328, 332, 333, 343) have been found exclusively in Hungary.

It was concluded that NCV strains cultured from water samples are heterogeneous even if the sample is taken from the same place of the same lake, or when taken from the same brook on 30 different occasions (Komárom county). The serotypes of NCV strains isolated from water samples in the counties investigated are mapped out (Fig. 1).

Results for toxin production of NCV strains are presented in Table VIII. These data are preliminary because of the inconsistent functioning of positive controls. To clarify the phenomenon, additional investigations are necessary. Living cultures of NCV strains dilated the ligated rabbit gut loop; 14 out of 20 inoculated segments proved positive independently of the origin of the strains (patient, healthy stools, water samples). Enterotoxigenic *E. coli* strain P99 isolated from pig was used as positive control. Five out of 7 segments showed fluid accumulation on being inoculated with P99 strain. Medium control failed to show positive reaction. Cell-free filtrate was also prepared from *E. coli* strain P99 and two NCV strains isolated from patient stools. While the positive control caused fluid accumulation in 9 out of 21 segments, the 2 NCV showed no positive reaction in either of the 8 segments examined.

Table VII

Distribution of serotypes of non-cholera vibrios isolated from water samples in Hungary between 1971—1976

Public health laboratory	Heiberg group	Serotypes																											
		11	12	13	14	15	16	17	18	19	20	21	22	27	28	29	30	31	33	39	40	42	43	44	46	48	50		
Borsod-Abaúj-Zemplén	I								1	2																			
	II														1									2					
Csongrád	I																												
	II										1																		
Fejér	I													1			1	1				1							
	II		1				1					2		1									2			1			
Győr-Sopron	I																		1										
	II	1			2	1					1										1			2					
Hajdú-Bihar	I	1		1	3	1		1		1				4			1					3							
	II	1												2			1					1							
Komárom	I				1																								
	II	2					1							1															
Pest (N.I.H.)*	I																			1									
	II					1					1		1													1	1		
Somogy	I															1						1							
	II	1				1						1					1	1				1		1					
Szabolcs-Szatmár	I	1			1						1		1		2								1						
	II																												
Vas	I																												
	II																							1					
Budapest	I																												
	II				1																								
Total	I	2		1	5	1		1	1	3	1		1	5	3		2	1	1	1		5	1						
	II	5	1		3	3	2				3	3	1	4	1	1		2			1	2	2	6		2	1		
	I-II	7	1	1	8	4	2	1	1	3	4	3	2	9	4	1	4	1	1	1	1	7	3	6	0	2	1		

Table VII (cont.)

Public health laboratory	Heiberg group	Serotypes																		Non- typable	Total					
		56	57	59	60	62	64	75	76	106	107	113	115	148	175	201	309	312	323			328	332	333	343	
Borsod-Abaúj- Zemplén	I									3															6	
	II																								3	
Csongrád	I							1														1			1	3
	II										5		1												7	
Fejér	I	1					1			3															6	15
	II			1		1																			4	14
Győr-Sopron	I																1		1						2	5
	II	1							1												1		1		2	14
Hajdú-Bihar	I												1	1		1	1	1							8	29
	II													1		2									2	10
Komárom	I		1																						1	3
	II								2					3											6	15
Pest (N.I.H.)*	I		1	2			1							1		2									3	11
	II																								1	6
Somogy	I																									2
	II				1																				8	16
Szabolcs-Szatmár	I														2										5	14
	II																								0	
Vas	I																									0
	II											1				2									3	7
Budapest	I																									0
	II																								1	2
Total	I	1	2	2	1	1	2	1		6			1	2	2	3	2	1	1			1			26	88
	II	1		1	1	1			3		5	1	1	4		4					1		1		27	94
	I-II	2	2	3	1	1	2	1	3	6	5	1	2	6	2	7	2	1	1	0	1	1	1		53	182

* National Institute of Hygiene, Budapest

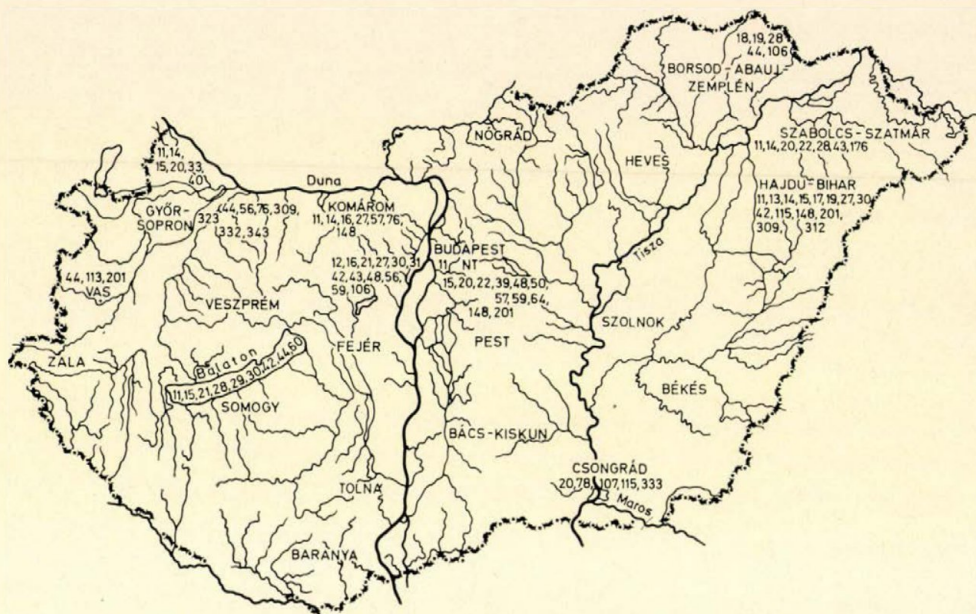


Fig. 1. Presence of serotypes of non-cholera vibrios in water samples in different geographic areas in the period 1971 to 1976

Table VIII

Presumable enterotoxicity of non-cholera vibrio strains for ligated rabbit gut loop

Strains	Broth			Tryptose agar		
	Range	$\bar{X}$	Positive/inoculated segments	Range	$\bar{X}$	Positive/inoculated segments
81938	2.2	2.2	1/1	0.03–0.2	0.1	0/5
69521	0.1–4.2	2.4	2/3	0.1	0.1	0/3
53371	0.3	0.3	0/1	.	.	.
38246	4.2	4.2	1/1	.	.	.
72473	3.4	3.4	1/1	.	.	.
57119	5.0	5.0	1/1	.	.	.
39802	0.8–4.5	2.8	2/3	.	.	.
39803	0.7–5.4	2.7	2/3	.	.	.
43999	0.4–4.3	2.1	2/3	.	.	.
44000	0.4–4.2	2.7	2/3	.	.	.
P99	0.1–6.3	2.9	5/7	0.1–4.5	1.46	9/21
Medium	0.1–0.3	0.2	0/4	0.1–0.2	0.15	0/5

Discussion

Two opinions are held as to the pathogenicity of non-cholera vibrios. One group reported them as the causative agent of cholera-like disease in India [26], gastroenteritis in Czechoslovakia [27] and diarrhoea in Sudan [28]. CHATTERJEE and NEOGY [29] implied a possible role of NCV in conjunction with other organisms such as *Aeromonas* and *Pseudomonas*. A group of workers state that NCVs are saprophytes found in various types of water and they rarely induce disease. BARUA and CVJETANOVIC [17] noted the ability of NCV to spread and questioned their ability to cause epidemics. SOLT [30] has reviewed the pathogenic role of NCV.

The initial problem encountered was the identification of *Vibrio*. The characteristics described by HUGH and SAKAZAKI [31] were used and the string test [32] and resistance to pteridine were also employed. All strains included in this report fit the basic pattern described for vibrios although two strains were string test negative and three were resistant to pteridine. SAKAZAKI *et al.* [33] too, found vibrios resistant to pteridine. Of the 200 strains investigated, 92 were Heiberg I, 108 were Heiberg II; the other 6 patterns failed to occur.

Further differentiation of the vibrios was achieved using the serotyping system described by SMITH and GOODNER [22]. Cultures which were identical in biochemical tests were subdivided serologically and then compared with similar strains in the *Vibrio* Reference Laboratory collection.

The isolates could be divided into three groups. The first was obtained from a total of 4459 stool specimens obtained from patients who had not travelled in cholera endemic or infected areas. Two stools (0.04%) yielded non-cholera vibrios. As compared to the occurrence of other pathogenic bacteria responsible for enteritis in Hungary in 1974, *Salmonella* were found in 2.4% and *Shigella* in 1.7% of the cases. In 6061 stool specimens from healthy individuals the incidence of vibrios was 0.03%; of *Salmonella*, 0.07%; and of *Shigella*, 0.1%.

Of 18 048 faecal samples of Hungarians returning from endemic or infected areas, NCV was isolated from 13 specimens of 8 individuals. This incidence approximated that for healthy, not travelled subjects. No infection was detected in the family members of these symptomless carriers.

NCV was isolated from 45.5% of the surface waters examined. Specimens taken from the same place on different occasions gave different results. For example, the Somogy county Public Health Station isolated 4 NCV from 15 water samples taken on May 25 and 14 from 15 water samples on July 5. Environmental conditions such as temperature may affect the number of organisms. It is believed that NCV and *Aeromonas* are part of the normal flora of surface waters and that their pathogenicity is low. If one considers

the accidental swallowing of approximately 50 ml of water while swimming, the lack of clinical symptoms in people swimming in such water would tend to support this concept.

Attempts to determine if some of the NCV isolates could cause accumulation of fluid in the ligated intestinal loops of rabbits have yielded equivocal results. At this time a statement regarding the pathologic mechanism(s) involved cannot be made.

From the above data it is evident that non-cholera vibrios are present in the environment and that these organisms are frequently ingested. Isolation of NCV from faeces does not necessarily mean that they are pathogens. They may represent contaminants or, in some individuals, part of the normal flora of the gut. Conversely, one should not ignore the possibility that these organisms, as has been shown by other authors, can cause gastroenteritis and other diseases.

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SELF-INOCULATION WITH MYCOBACTERIUM TUBERCULOSIS WEISZFEILER W-115

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(Received April 11, 1978)

After intracutaneous self-inoculation with 0.05 mg (wet weight) of *Mycobacterium tuberculosis* W-115 containing approximately 600 000 viable units, the local reaction was observed during a period of 9 weeks. No adverse effects or general reaction were observed. From the lesion the W-115 strain was recultivated. It was labelled W-115(78)ST vaccine strain and is used for further studies. On the basis of experiments on laboratory animals, monkeys, human new-borns and adults, the strain W-115 is considered as safe for anti-tuberculous vaccination as the strains *Mycobacterium bovis* BCG and the attenuated "MP" strain of *Mycobacterium microti* OV 166.

The *Mycobacterium* strain W-115 was isolated in Tashkent in 1953 from the blood of a tuberculous patient from whose sputum a virulent strain of *Mycobacterium tuberculosis* had been cultured. The avirulence for the guinea pig and rabbit of the W-115 strain prompted WEISZFEILER [1] to use it for antituberculous vaccination of humans after experiments in rhesus monkey had demonstrated its attenuated virulence. The tuberculous changes were confined to the site of infection and no dissemination was observed. In 1965, trials of the strain in rhesus monkeys revealed a higher immunogenicity than of BCG [2]. In 1965 and 1972, conferences were held [3, 4] in Budapest summarizing current experiences and the first observations on vaccination of children in Tashkent. In 1974 twenty new-borns were vaccinated with BCG vaccine and with the new lyophilized W-115 vaccine prepared by the Institute of Sera and Vaccines in Tashkent [5]. The vaccination dose was 0.05 mg/0.1 ml, containing approximately 600 000 viable units. After 3 and a half years' observation in Samarkand [6], the status of the vaccinated children was normal, no signs of adverse local or general reactions were noted.

These results and some self-observations of subjects who had been for many years tuberculin negative reactors [7] have justified to conclude to the safety of *M. tuberculosis* W-115 as a new vaccine strain for humans.

Material and methods

For the experiments an original lyophilized W-115 vaccine prepared at the Institute of Sera and Vaccines in Tashkent was used. The lyophilized material was a fine homogeneous suspension containing small aggregates of short acid-fast granulated or coccoid rods (Pictures

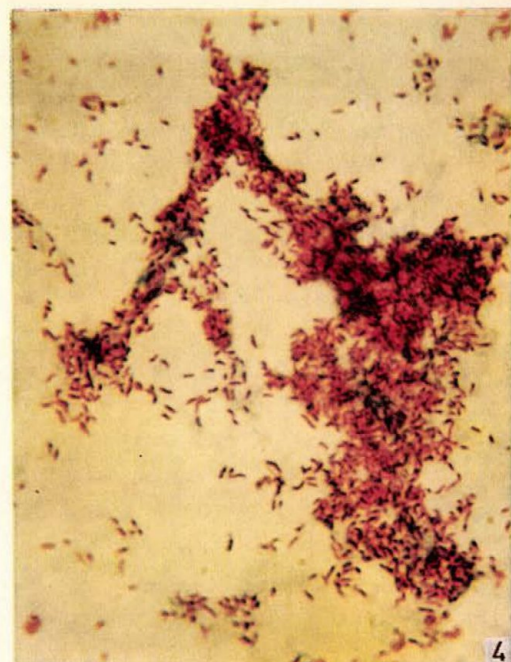
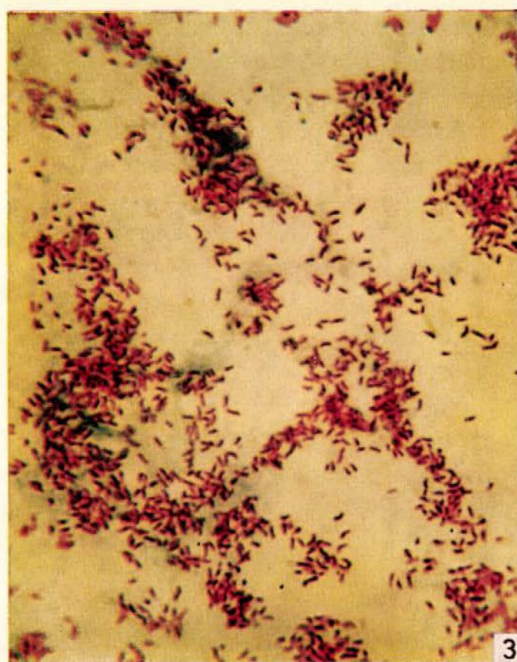
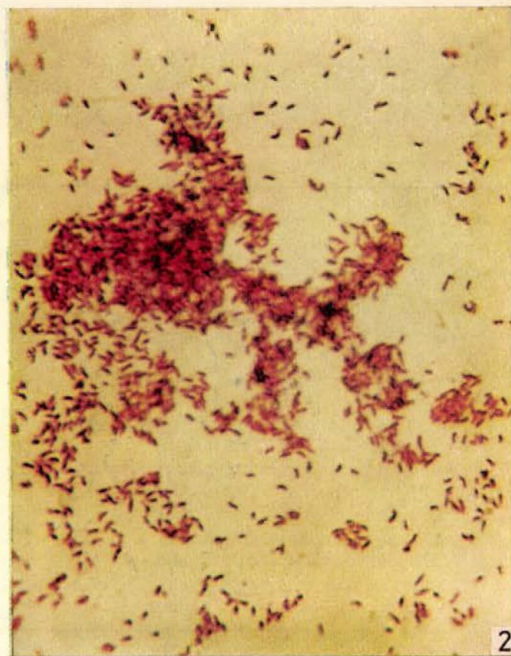
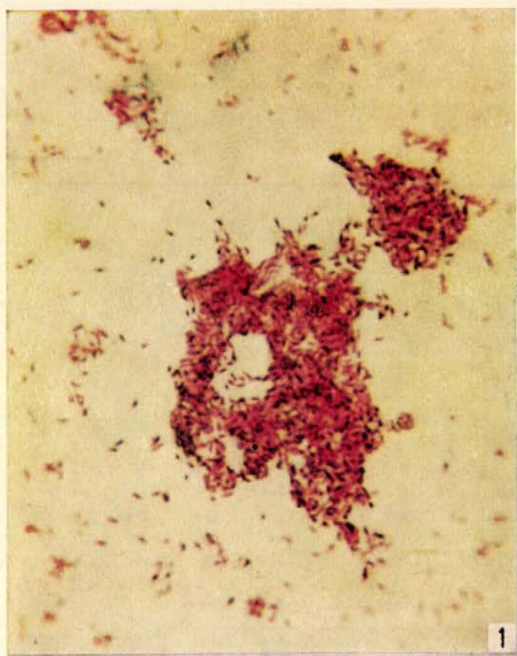


Plate 1. Morphology and acid-fastness of *Mycobacterium* species W-115 (suspension of a lyophilized W-115 vaccine containing 1 mg/1 ml)



Plate 2. The course of early local reaction after intracutaneous inoculation of W-115 vaccine (0.05 mg containing approx. 600 000 viable units.) In Picture 5 author's tuberculin reaction before vaccination after injection of 2 TU PPD RT 23

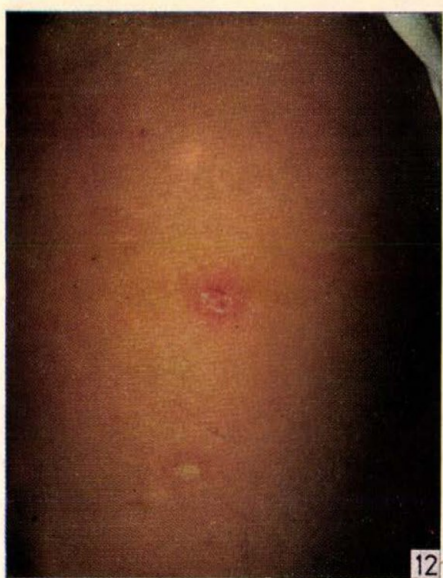
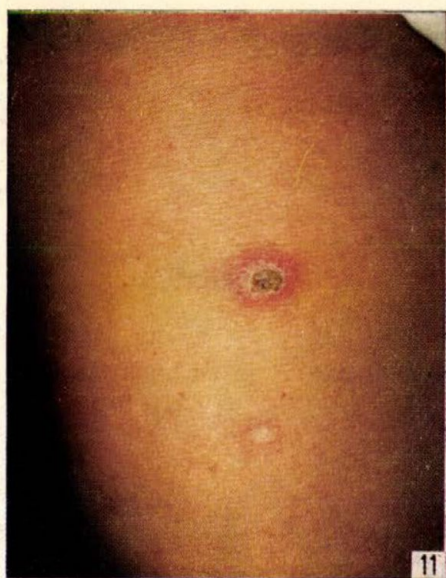
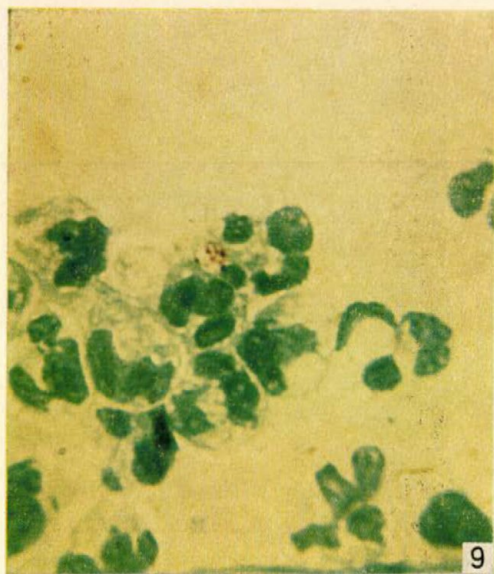


Plate 3. The course of late W-115 local reaction with a complete healing after 9 weeks following the intracutaneous injection

1-4, Plate 1). The vaccine was reconstituted in 1 ml of saline and 0.1 ml of it was injected intracutaneously in the deltoid region of the left upper arm.

Before the vaccination a tuberculin test with 2 TU PPD RT 23 was made on 9.11.77. The local reaction was read after 48 hr and showed a 17×17 mm infiltrate with a 10×10 mm red halo (Picture 5, Plate 2). The vaccination was carried out on 15.11.77 and the local reaction was observed up to complete healing on 23.1.78 (Pictures 6-12, Plates 2 and 3).

Discussion

The local reaction is typical for a tuberculin positive person and can be characterized in general as the Koch phenomenon. After 24 hr following the W-115 vaccination a local inflammation 12×16 mm in size appeared with a small centre indicating the penetration of the injection needle (Picture 6). Picture 7 taken one week later, on 21.11.77, shows that the local reaction measured 9×10 mm. On the periphery it is well circumscribed and in its centre a 2×2 mm pustule has developed. The difference between the two reactions was probably caused by the mechanical trauma of the injection leading to the dissociation of epithelial cells followed by a reparative process. A significant difference in the character of the reaction was noted on 27.11.77 when suddenly an increasing redness and pain, then within 24 hr in the centre of the inflammatory area a pustule appeared which almost immediately burst releasing a few drops of yellowish pus (Picture 8). The secretion was collected and inoculated on Löwenstein-Jensen and Šula's liquid medium. In the smear accumulation of neutrophils and mononuclear cells and a few clumps of phagocytosed partly destroyed acid-fast rods were found (Picture 9). The following course of the reaction is shown in Picture 10 (1.12.77), Picture 11 (20.12.77) and Picture 12 (23.1.78). After complete healing of the postvaccinal lesion a new tuberculin test with 2 TU PPD RT 23 was performed on 29.3.78. No increase in tuberculin sensitivity had occurred, the induration measured 17×17 mm.

The whole course of my own tuberculin allergy was followed systematically since 1962; Fig. 1 shows my changing sensitivity to tuberculin during the period 1962 to 1967. Afterwards a certain leveling lasting from 1967 to 1971 followed after self-vaccination with the attenuated Murinus Prague "MP" strain of *M. microti* but no increase after the W-115 vaccination and no booster effect of repeated tuberculin testing were observed.

It would be far reaching to explain all these long term variations of my tuberculin tests being during my laboratory work daily exposed to mycobacterial aerosols and even to contact infections leading to repeated absorption of tuberculous antigens maintaining a high level tuberculin allergy but never exceeding a 20 mm size induration. As soon as this level has been achieved, which is very likely genetically conditioned, further resorption of mycobacterial aerosols is without any allergenic effects.

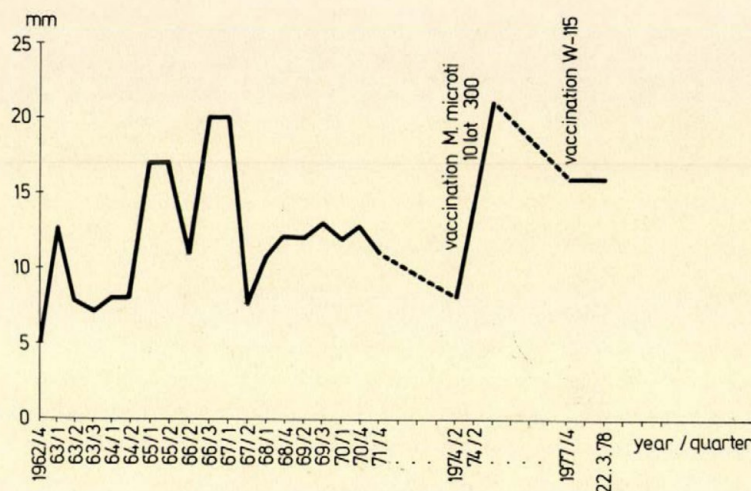


Fig. 1. Author's own tuberculin allergy to 2 TU PPD RT 23 during the period 1962 to 1978

From the pus of the local lesion the W-115 strain was recultivated on Löwenstein-Jensen and Šula's media. Now it is maintained on Sauton medium and the first passage carried out in January, 1978, was labelled W-115 (78)ST and is used for further experiments.

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NOTE

EFFECT OF DEEP FREEZING ON THE RESULT OF BLOOD CULTURES

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From citrated blood samples left to stand in the deep freeze at -20°C for 12–14 hr prior to culturing, *Staphylococcus aureus* was recovered more effectively than from the control cultures.

Several papers dealt with the improvement of blood cultures by using hirudin, polyanetholsulphonate [1], oxalate or citrate [2, 3] as anticoagulants and glucose–trypsin broth [4] as medium. For single flask cultures the dilution method to avoid contamination and the prolongation of the incubation time were recommended [5]. Aerobic–anaerobic culturing in closed flask with improved medium and prolonged incubation time [6] as well as rapid membrane filter technique [7] have also been described.

Records for blood cultures show a predominance of Gram-negative rods and a decreased incidence of *Staphylococcus aureus*. In the present paper a simple method is described ensuring an effective cultivation of this organism.

Methods. One hour before the patient's temperature was expected to reach the maximum, a 10 ml sample (1 part 3.5% citrate + 3 parts blood) was obtained from the medium cubital vein. Half of the sample was refrigerated at -20°C for 14–16 hr, the other half was inoculated at 1 ml portions into 5 Levinthal broth tubes. Next days the frozen sample was inoculated similarly. On the 3rd and 5th days aerobic and microaerophilic subcultures were made from each tube on blood and chocolate agar plates.

Model experiments were performed by adding graded dilutions of a 24 hr broth culture of *S. aureus* to the citrated blood sample taken from a healthy person. The mixtures were left to stand at 37°C for 10 and 20 min, then halved and treated as described above. The average staphylococcal count obtained in three repeated experiments was used for evaluation.

Results. A total of 660 blood samples were taken from 198 patients mainly with endocarditis, carditis and pneumonia and less frequently with sepsis, pyrexia and cystopyelitis. Results for the frozen and usual samples are shown in Table I.

Table I
Result of blood cultures

Total No.	Bacteria not cultured	Pathogenic bacteria cultured			Contaminated frozen sample + usual sample
		frozen sample + usual sample	frozen sample	usual sample	
660	585	27	28	10	10

The distribution of bacteria cultured from the samples is shown in Table II. From 9 patients the same organisms were isolated on 2 to 5 occasions. The result of model experiments is presented in Table III.

Table II
Distribution of organisms cultured from blood samples

Organisms	Frozen sample	Usual sample	Both samples
<i>Staphylococcus aureus</i>	21	6	19
<i>Micrococcus</i>	1	2	2
Alpha-haemolytic <i>Streptococcus</i>	6	1	4
<i>Candida</i> *	—	1	1
<i>Acinetobacter anitratus</i>	—	—	1
<i>Corynebacterium</i>	—	—	1
Total	28	10	28

* Verified at autopsy.

Table III
*Model experiment for the recovery of *S. aureus* from blood samples*

Sample	Recovery of <i>St. aureus</i> added at dilution and colony forming units per ml			
	10 ⁻⁶ 490	10 ⁻⁷ 280	10 ⁻⁸ 44	10 ⁻⁹ nil
Usual	+	+	+	—
Frozen	+	+	+	—

Discussion. The usual method and the deep freeze technique described in the present paper gave different results for blood samples. Cultures started from frozen blood samples were more effective especially for patients suffering from clinically verified staphylococcal diseases.

Although model experiments showed no difference between the two methods, they indicate that freezing did not influence the survival of

staphylococci in the samples. The higher resistance to cooling as compared to other bacteria, of *Staphylococcus*, *Micrococcus* and alpha-haemolytic *Streptococcus* has been described [6]. As to the higher recovery of staphylococci from patients, it may be assumed that freezing promotes the release of the bacteria from leucocytes and allows in this manner a less inhibited multiplication. Alterations in the plasma constituents may also take place.

In view of the simple technique, the method may be recommended for routine use.

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NOTE

MIDDLE EAR INFECTION DUE TO *VIBRIO*
ALGINOLYTICUS. BACTERIOLOGICAL
CHARACTERIZATION

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A 47-year-old male suffering from chronic purulent ear discharge was investigated bacteriologically in autumn 1977. The isolated strain was identified as *Vibrio alginolyticus*. The fact that the patient spent his vacation at a Black-Sea resort every year strongly pleads for a direct inoculation of the halophilic *Vibrio*.

Widely distributed throughout the world, the *Vibrio* genus of bacteria remains worthy of attention due to the fact that the flame of cholera is still flickering and the so-called non-agglutinable (NAG) vibrios belonging to halophilic or non-halophilic groups are a common cause of intestinal disorders (diarrhoea, food poisoning) and extraintestinal infections [1]. Halophilic vibrios occur in most sea waters (brackish and pelagic), sea fauna (fish, shellfish, molluscs, crabs) and sea mud.

Despite the frequency of intestinal disorders caused by halophilic vibrios, extraintestinal infections have rarely been reported [2-5]. This paper presents details of a patient with extraintestinal *Vibrio alginolyticus* infection.

Report of a case. A 47-year-old male suffering from a chronic purulent discharge from the right ear sought medical attention in early autumn 1977. The history revealed polyps in the injured and consecutively infected external auditory canal with myringitis, purulent otitis media and chronic mastoiditis cured by mastoidectomy some years ago. Pain, impaired hearing, drum perforation through which pus flowed, were present.

The patient spent a fortnight vacation on the Black-Sea shore every year, where halophilic vibrios were isolated from water and marine fauna since 1973 (RUSU, personal communication).

From a swab taken from his right ear a Gram-negative, slightly curved organism was isolated on 5% sheep blood agar (strain number 1191).

Identification was performed according to a selected differential set of tests, as shown in Table I.

Results and discussion. On 5% sheep blood agar the isolated strain formed large and circular low convex, mucous, opaque, confluent colonies; after 18

hr at 37 °C, it resembled mucous forms of *Escherichia coli*. On TCBS medium the culture formed mucous, sucrose-fermenting (yellow) colonies. Swarming phenomenon was positive on sheep blood agar and nutrient agar, if the Petri dishes were subsequently maintained at 22 °C (room temperature) for 2 to 3 days.

The physiological and biochemical characteristics of the strain (No. 1191) are listed in Table I. It was identified as *V. alginolyticus* (motile, fermentative in glucose O-F Hugh-Leifson test, oxidase, gelatinase, lysine and ornithine decarboxylase positive, sucrose positive, arabinose negative, growth in 10–12% NaCl peptone water, positive swarming phenomenon).

Table I
Physiological and biochemical characteristics of strain 1191

Characteristics	Results	Characteristics	Results
Gram stain	—: slightly curved	Sucrose	+
Growth in peptone water		Mannitol	+
with 0% NaCl	—	Adonitol	—
with 0.5; 3; 5% NaCl	+	Inositol	—
with 8; 10; 12% NaCl	+	Mannose	—
Pellicle formation	+	Rhamnose	—
Indole production	±	Arabinose	—
H ₂ S production (TSI)	—	Sorbitol	—
Methyl red	±	Dulcitol	—
Voges-Proskauer	—	Salicin	—
Simmons citrate	±	Maltose	+
Phenylalanine deaminase	—	Trehalose	+
Oxidase (Kovács)	+	Fructose	+
Motility	+	Xylose	—
Haemolysis (sheep)	—	Cellobiose	—
Nitrate reduction	+	Raffinose	—
Urease	—	Lysine decarboxylase	+
Malonate	—	Ornithine decarboxylase	+
Gelatin liquefaction	+	Arginine dihydrolase	—
Catalase	+	Swarming phenomenon	+
Acid from glucose	+	String test	+
Gas from glucose	—	Sensitivity to O/129	+
Glucose O-F test	F		
Lactose	—		

3% NaCl was added to all media.

F = fermentative in the Hugh-Leifson test.

According to SAKAZKI [6] the swarming ability is a fundamental physiological property of a bacterial species, and an essential difference between the two biotypes of *Vibrio parahaemolyticus*. The strain belonged to Heiberg's group II. It was sensitive to tetracycline, chloramphenicol, erythro-

mycin, colistin, nalidixic acid (300 μ g), nitrofurantoin, neoxazol, furazolidine, rifampicin and 0/129 vibriostatic agent.

V. alginolyticus belongs to the family *Vibrionaceae*, genus *Vibrio*. Until 1965, it was designated subgroup 2 of *V. parahaemolyticus*. In 1968, SAKAZAKI carried out a second comparative study of the biotype 1 (*V. parahaemolyticus*) and biotype 2 (*V. alginolyticus*). As shown in Table II, *V. alginolyticus* produced acetoin, fermented sucrose, grew well in 10% NaCl, peptone water, showed the swarming phenomenon, while *V. parahaemolyticus* failed to ferment sucrose, to grow in peptone water containing 10% NaCl and to reveal any swarming ability.

Table II

Dissimilar characteristics of V. parahaemolyticus and V. alginolyticus

	Biotype 1 (<i>V. parahaemolyticus</i>)	Biotype 2 (<i>V. alginolyticus</i>)
Growth in 10% NaCl	—	+
Voges-Proskauer reaction	—	+
Sucrose fermentation	—	+
Swarming ability	—	+
Acid from arabinose	d	+
Methyl red	+	—
TCBS medium	green colonies	yellow colonies

Because of the wide distribution of some *Vibrio* species in sea-water, mud and fauna and the extensive use of beaches, a reappraisal of the importance of non-cholera vibrios in cholera free areas is necessary due to their participation as intestinal and extraintestinal potential pathogens.

Extraintestinal infections are due either to seeding after gastro-enteritis and sepsis (*V. parahaemolyticus*) or to direct inoculation [4, 5] as it is strongly suggested in the present case (90% *V. alginolyticus* mixed with *Staphylococcus albus* colonies).

McSWEENEY and FORGAN-SMITH [7] recently reported the first isolation in Australia of *V. alginolyticus* from an infected wound (acute otitis media with drum perforation and purulent discharge after a fall from the surfboard at a costal resort).

A more accurate and precise identification of oxidase positive strains would probably reveal a greater incidence of non-cholera vibrios as aetiological agents of extraintestinal wounds.

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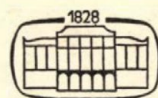
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ABSORPTION OF COLOSTRAL IMMUNOGLOBULINS IN SUCKLING PIGLETS

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(Received January 4, 1978)

The three portions of the small intestine of 18 neonatal piglets were examined by using the so-called scroll technique evolved by the authors and in cross sections. The chemical constitution and the immunoglobulin content of undigested protein droplets being under absorption were studied. An intensive absorption of immunoglobulins was demonstrated in the body of each cuticulated cylindric epithelial cell of the mucosa covering the jejunum and the proximal two-third of the ileum. Absorption was the most intensive, though variable by area, in the jejunum. It was demonstrable 4 hr after birth and reached the highest intensity between 8 and 12 hr, then tended to decline. In the protein droplets alpha-amino acids, neutral mucopolysaccharides and protein-bound Ca^{2+} were detected. Alkaline phosphatase activity was demonstrated around the droplets. Immunofluorescence and immunoperoxidase reactions proved the presence of immunoglobulins, first of all of IgG, in droplets. Electron microscopically, the microvilli of the involved epithelial cells were swollen, sometimes neighbouring ones were pushed away from each other; in the same areas, an electron-dense substances (colostrum) covering the enterocytes was streaming through the tubulovascular network of cells to the perinuclear region, where it appeared in spherical droplets.

The epitheliochorial placenta of swine does not allow maternal immunoglobulins to reach the fetus *via* the blood circulation. On the first days of extrauterine life, piglets, take up antibodies from the colostrum. The absorption of colostrum proteins is, however, disturbed in enteric conditions, most of which are caused by *Escherichia coli* or viruses. The literature is abundant in reports dealing with these conditions but little has been published on their histological diagnosis and differential diagnostics [1–6] and on the corresponding morphological features in normal piglets [7–11]. The morphological changes accompanying the absorption of undigested protein have been investigated in several intestinal portions mainly in laboratory animals [12–21].

The morphological characteristics of the absorption of undigested protein in the normal suckling piglet as well as the intestinal localization and temporal course of the process are the subject of the present report.

Materials and methods

The absorption of undigested large molecular weight proteins was examined in the small intestine of 18 neonatal piglets. Piglets were exsanguinated 4, 8, 12, 17, 24, 51, 72, 144 and 156 hr after birth. Tissue samples were taken for histochemical, immunological and electron-microscopic studies from the proximal and distal parts of each of the three portions of the small

intestine and from the middle third of the duodenum. The residual part of the small intestine was processed by the scroll technique for light microscopy (Fig. 1). The small intestine, still of body temperature, was cut into 20 cm portions beginning from the pylorus, opened along the mesenteric insertion and extended on glass plates. A paper strip as wide as the intestinal strip was soaked in saline and placed on the preparations. These were rolled up like tapes, loosely tied with thread and placed into 10% formalin. A piece of gut 1.5 cm in width, from the proximal part of each portion, was fixed for examination in cross section. Before embedding in paraffin, the thread was cut through, the scroll was extended and the filter paper was removed. Then the intestinal segment, due to its elasticity, rolled up again.

For general orientation, Mayer's haemalaun-eosin, for staining of mucus the haemalaun-mucicarmine and the PAS reaction, for elective demonstration of protein droplets the polychrome staining of Farkas and Mallory were applied. Elastic fibres were demonstrated with resorcin-fuchsin.

Samples taken for histochemical and immunological tests were either kept unfixed at -20°C until sectioned or were fixed in formalin. The formalin-fixed samples were embedded either in paraffin or according to SanMarie's acetone-cold method [22].

Undigested protein droplets were examined by the following histochemical procedures: alloxan-Schiff method for alpha-amino acids; PAS, alcian blue-PAS, Hale and Hale-PAS methods for polysaccharides; metachromatic toluidine blue staining; Kossa's reaction for demonstration of $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 ; alizarin-red S reaction for protein-bound Ca^{++} . Nucleic acid, neutral fat and alkaline phosphatase were detected by the methylgreen-pyronine, Fettrot and azo-binding reactions, respectively.

FITC and immunoperoxidase conjugates were prepared from an anti-pig-colostrum serum. This was raised in rabbits as follows. Eight rabbits were injected with the defatted casein-free globulin fraction of colostral serum emulsified in complete Freund's adjuvant. Of the first dose, 0.1 ml/rabbit was injected into the foot pads and 0.6 ml, divided into two equal volumes, was injected subcutaneously at two different sites. Two weeks later, 2×0.5 ml emulsion was injected. The rabbits were exsanguinated after another two weeks. Sera having a titre over 1 : 10 000 were used for preparation of conjugates.

Conjugation with FITC and the immunofluorescence tests were performed as usual [23, 24]; specificity of the fluorescence was controlled by pretreating preparations with non-conjugated anti-pig-colostrum serum (extinction of fluorescence).

For immuno-peroxidase tests, the IgG fraction of the anti-pig-colostrum serum was purified by column chromatography on Sephadex G-150 gel and labelled with peroxidase as described by NAKANE [25]. The preparations were washed with PBS, covered with peroxidase-labelled serum and incubated in a wet chamber at 37°C for 30 min. The preparation thus treated were rinsed in PBS three times and placed in diaminobenzidine (DAB) developer for 50 s. Finally, after repeated rinsings in PBS, nuclear staining was performed with kernechtrot.

For electron microscopy, tissue samples from different intestinal sections were prefixed in Karnowsky's solution and postfixed in 1% OsO_4 . Dehydrated tissue fragments were embedded in Durcupan resin. Ultrathin sections were shadowed with uranyl acetate and lead nitrate.

Results

Absorption of large-molecule proteins could not be observed in the duodenum, except in the part containing Brunner's glands and the cuticulated cylindrical epithelial cells (enterocytes) of several villi located in the subsequent 20 cm portion. In the following parts of the small intestine, an intensive absorption was demonstrated in every mucosal cell till the first two-thirds of the ileum. Undigested large-molecule protein was absorbed only in the enterocytes covering intestinal villi. We failed to observe characteristic phenomena in Lieberkühn's crypts.

Absorption of protein was observed as early as 4 hr after birth (Fig. 2) and it was the most intensive between 8 hr and 12 hr (Figs 3 and 7). Absorp-

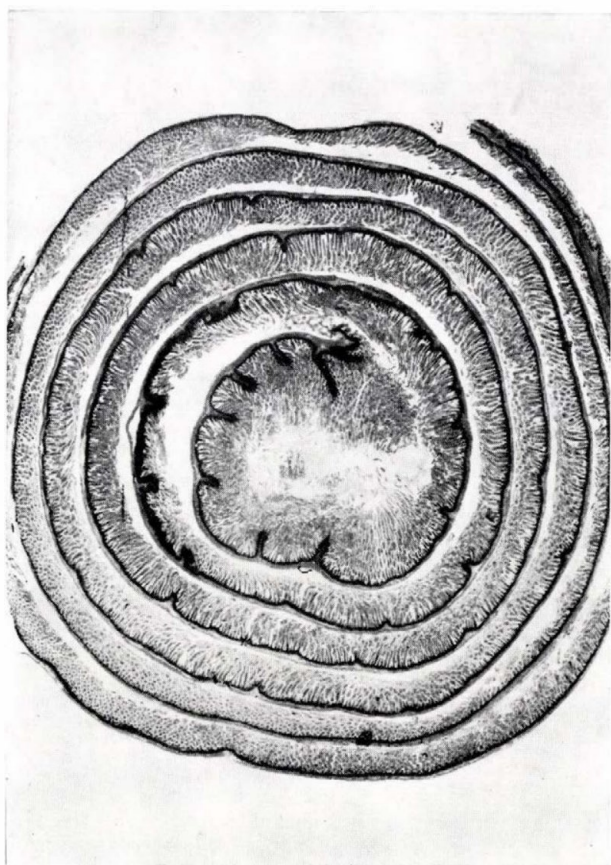


Fig. 1. Histological section from a rolled-up jejunal section 20 cm in length. The suckling piglet was exsanguinated 8 hr after birth. Haemalaun-eosin stain, low-power

tion of undigested protein tended to decline at 24 hr; in the 51st hr only enterocytes covering the apex of villi at the proximal end of the ileum were involved. Later, the process was confined to scattered epithelial cells.

Protein under absorption appeared as homogeneous eosinophil droplets. Initially, small droplets appeared in the enterocytes. Later, droplets coalesced until cells were filled with a single eosinophilic mass. Some cells became barrel-shaped (Fig. 4). Protein droplets were seen in basal location in epithelial cells immediately beneath the cuticula and also in the lymphatics of intestinal villi (Fig. 7).

Polychrome staining showed the intracellular protein droplets blue, red or golden-yellow in colour while those in the intestinal lumen stained a pale blue. Some droplets had a bright red centre and blue edges. Twelve and 24 hr after birth, most of the droplets appeared golden-yellow or red, only few stained

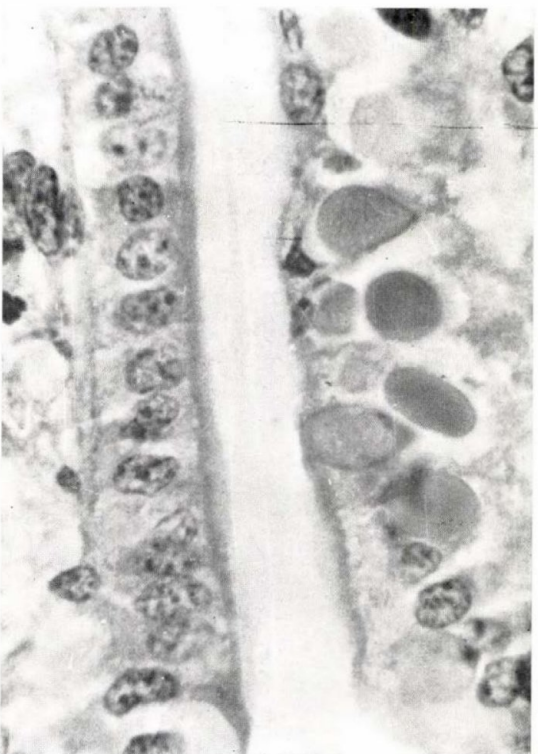


Fig. 2. Jejunum of a 4-hour-old suckling piglet. Absorbed protein droplets are seen in some of the enterocytes. Haemalaun-eosin $\times 600$



Fig. 3. Jejunum of a 12-hour-old suckling piglet. Intensive protein-droplet absorption in every enterocyte. Haemalaun-eosin $\times 95$

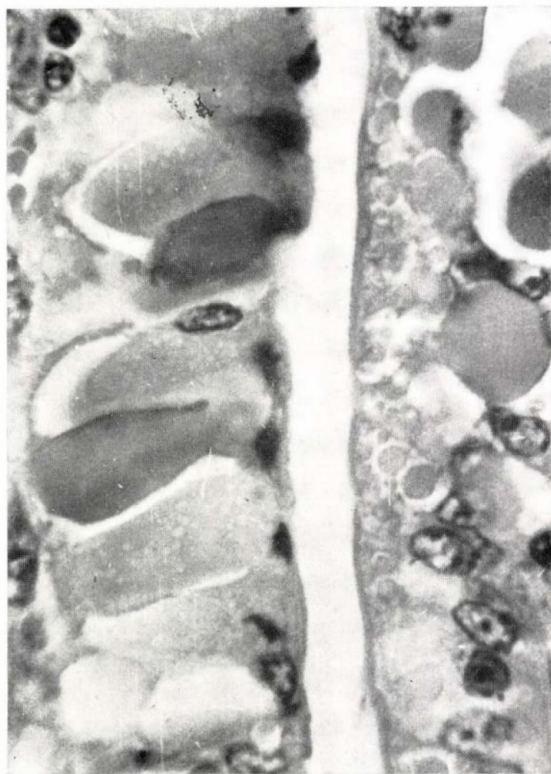


Fig. 4. Jejunum of a 12-hour-old suckling piglet. The body of many enterocytes is filled with a homogeneous mass of fused protein droplets. Note shrunk nuclei located laterally or beneath the cuticula. Haemalaun-eosin $\times 1400$

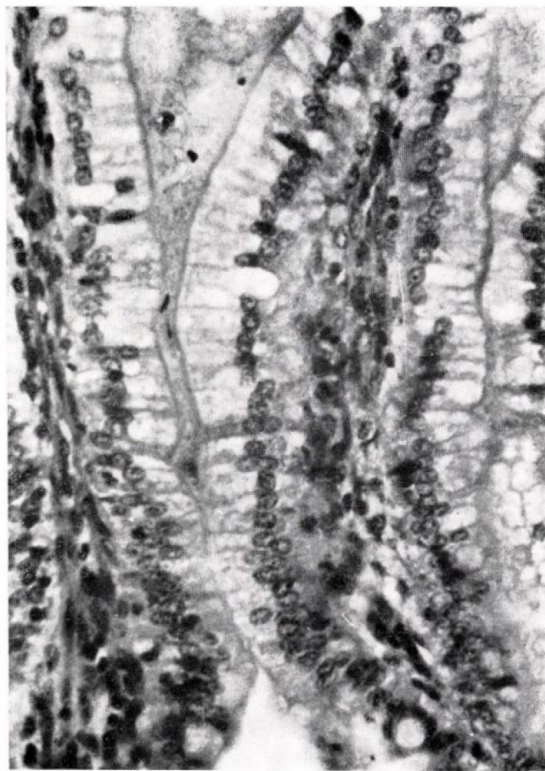


Fig. 5. Jejunum from a 72-hour-old suckling piglet. No protein droplets in the cavernous cytoplasm of mucosal epithelial cells. Nuclei in basal location. Haemalaun-eosin $\times 240$

blue. The epithelial cells of the jejunum contained protein droplets at the highest frequency, though this frequency varied by area.

Initially, the nucleus of the active enterocyte, like that of the inactive enterocyte, was located basally; then the protein droplet passing along the cell pushed the nucleus to the central, and still later to the apical, part of the cell (Fig. 4). As the absorption decreased in intensity, the body of the cell became cavernous and the nucleus took up a basal location again (Fig. 5). Still later, regenerating protoplasm filled the body of the cell (Fig. 6).

In absorbed droplets, a positive alloxan-Schiff reaction (Fig. 8) indicated protein-like substances containing alpha-amino acids. The droplets appeared PAS positive (Fig. 9), alcian blue negative and Hale negative. The metachromatic toluidin blue reaction showed the droplets orthochromatic (Fig. 10). All these indicated the presence in the droplets of neutral mucopolysaccharides. We failed to detect $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 by Kossa's reaction, but protein-bound Ca^{2+} was demonstrable with alizarin-red S. The droplets did not stain with methylgreen-pyronin, i.e. they did not contain nucleic acid. Using Fettrot stain we detected fine neutral fat granules around the intracellular protein droplets. The azo-binding reaction indicated alkaline phosphatase activity in the cuticula of epithelial cells covering the initial parts of the duodenum, i.e. cells not participating in the absorption (Fig. 11); elsewhere in the small intestine, alkaline phosphatase activity was detected around intracytoplasmic protein droplets (Fig. 12).

Immunofluorescence of slightly variable intensity was demonstrated with the pig colostrum antiglobulin conjugate in the protein droplets in enterocytes located in the distal part of the duodenum, along the jejunum and in the first two-thirds of the ileum. The bright fluorescence mostly extended to the whole cell; the rest displayed a bright peripheral and weak central fluorescence (Fig. 13). No fluorescence was observed in preparations pretreated with non-conjugated anti-pig-colostrum serum.

The immune peroxidase reaction appeared as an intensive, apparently homogeneous colour reaction in the involved enterocytes (Fig. 14).

Electron micrographs showed swelling of the microvilli of the cells participating in protein absorption; at some places neighbouring microvilli seemed to be pushed away from each other (Fig. 15). In the corresponding areas, highly electron-dense substance (colostral milk) was streaming from the cell surface into the intestinal cells (Fig. 16). The dense substance entered the tubulovesicular network and appeared perinuclearly as oval or spherical bodies (Fig. 17). Fusion of these bodies resulted in bodies gradually growing in size in enterocytes migrating towards the lymphatics. In the protoplasm of these enterocytes, the rough endoplasmic reticulum became abundant and free ribosomes were growing in number; on the other hand, the cytoplasm was poor in mitochondria.



Fig. 6. Jejunum of a suckling piglet 156 hr after birth. The vacuoles in the body of epithelial cells is gradually filled by the cytoplasm. Haemalaun-eosin $\times 1400$

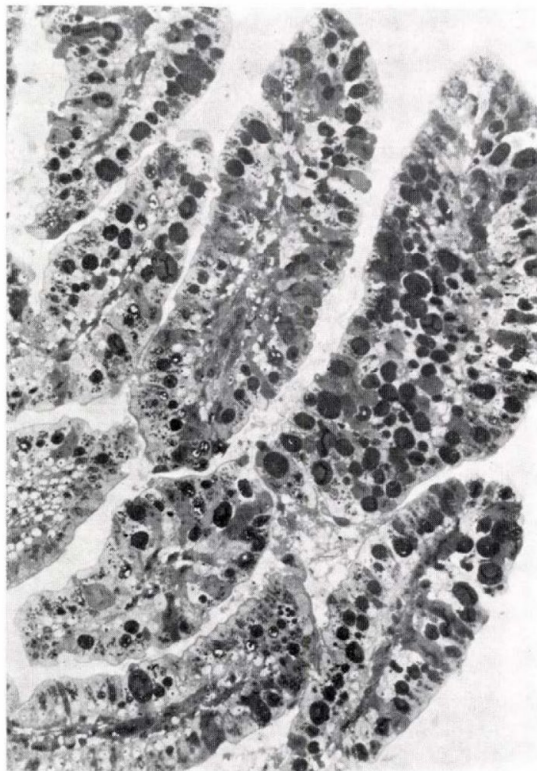


Fig. 7. Jejunum of a suckling piglet 12 hr after birth. Protein droplets in epithelial cells covering the mucosa and in the lymphatics of intestinal villi. Semithin section, toluidine blue. $\times 250$

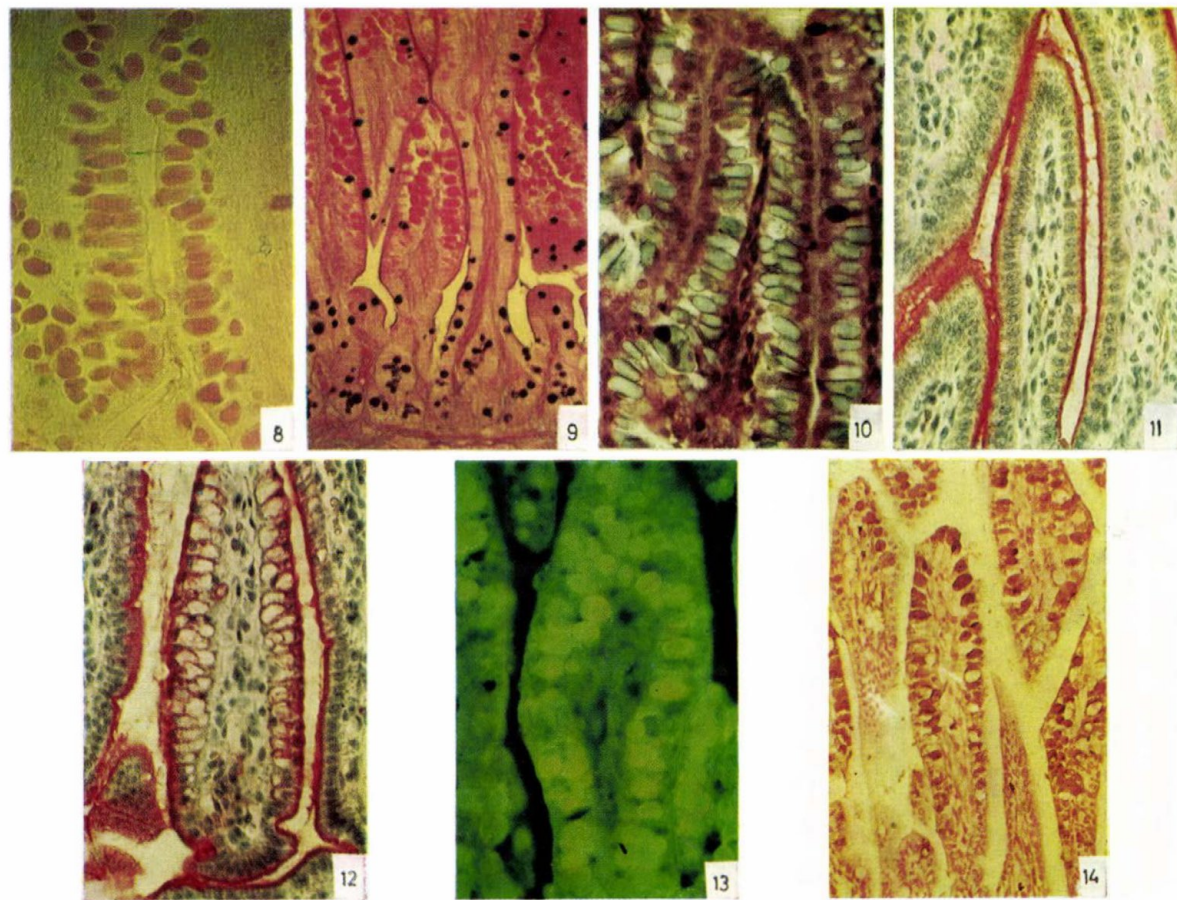


Fig. 8. Jejunum of a suckling piglet 17 hr after birth. The protein droplets in the enterocytes in the villi of the mucous membrane stain orange with alloxan-Schiff. $\times 600$. Fig. 9. Jejunum of a suckling piglet 17 hr after birth. Alcian blue-PAS. The absorbed protein stains red (PAS positive), the acid mucosubstance in goblet cells is alcianophilic. $\times 100$. Fig. 10. Jejunum of a suckling piglet 12 hr after birth. Metachromatic toluidine blue. The absorbed protein droplets stain orthochromatically, appearing greenish-blue. The mucus in goblet cells stains metachromatically red. $\times 240$. Fig. 11. Proximal portion of the duodenum of a suckling piglet 12 hr after birth. Azobinding reaction. The cuticula of the enterocytes covering the mucosal villi shows alkaline phosphatase activity. $\times 120$. Fig. 12. Jejunum of a suckling piglet 12 hr after birth. Azobinding reaction. In the enterocytes of mucosal villi, besides the cuticula, the cytoplasm around the absorbed protein droplets shows alkaline phosphatase activity. $\times 240$. Fig. 13. Jejunum of a suckling piglet 12 hr after birth. Absorbed protein droplets show an intensive fluorescence with FITC conjugate of the anti-globulin. In some cells the peripheral, in others the central, fluorescence is more intensive. $\times 600$. Fig. 14. Jejunum of a suckling piglet 12 hr after birth. Absorbed protein droplets give a positive reaction with the peroxidase conjugate of the IgG fraction of the anti-globulin serum. $\times 100$.

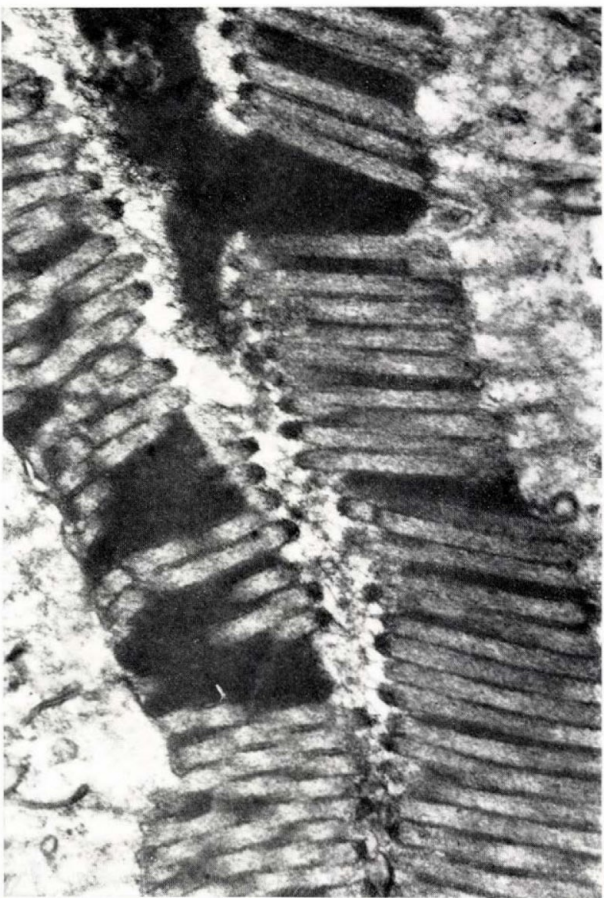


Fig. 15. Jejunum of a suckling piglet 12 hr after birth. Highly electron-dense colostrum covering the microvilli of enterocytes is streaming into the enterocytes. Note swollen microvilli being pushed away from each other. $\times 31\ 000$



Fig. 16. Jejunum of a suckling piglet 17 hr after birth. Electron-dense colostrum is streaming from the intestinal lumen between microvilli into the dilated tubulovesicular system of the cylindrical epithelial cell. $\times 31\ 000$



Fig. 17. Jejunum of a suckling piglet 17 hr after birth. In the enterocyte, the absorbed colostrum forms highly electron-dense droplets in the perinuclear area. $\times 15\,000$

Discussion

The aim of the present study was to gain information by light and electron-microscopic investigations on the chemical constitution and immunoglobulin content of protein droplets being under absorption in enterocytes of neonatal suckling piglets.

The histological investigations were considerably facilitated by the scroll technique, a method evolved by us to study all epithelial cells of the villi of individual intestinal sections in a single plane along the whole small intestine. Thus we have been able to show exactly the sites of the absorption of protein droplets in the small intestine of suckling piglets exsanguinated on different postnatal days and to describe the accompanying morphological pictures. Ear-

lier investigations [7, 20, 24] had involved only small areas of the small intestine.

Proteins of large-molecular weight were most intensely absorbed from the jejunum and the first two-thirds of the ileum till the 8th to 24th hr after birth.

STANLEY *et al.* [26] did not mention the duodenal section from which their samples had been taken. Based on the present findings we assume that they had taken the samples from areas near the jejunum; otherwise considerable absorption would not have been seen.

The morphological changes observed during the absorption of protein droplets were variable even in adjacent enterocytes. Some epithelial cells contained only small droplets while neighbouring ones were filled with a mass of protein-like substance. The nucleus of the involved enterocytes changed typically its place during protein absorption. We have failed to find similar observations in the literature available.

Since only few epithelial cells of the duodenal villi and other enterocytes displayed degenerative changes, it seems unlikely that the majority of the involved cells should perish. Our morphological observations are in accordance with physiological findings [7, 27].

We found no data in the literature on the histochemical and immunological characteristics of the undigested protein droplets present in the epithelial cells covering the small intestinal mucosa of suckling piglets.

The protein-like substances present in the droplet under absorption were found to contain alpha-amino acids. Besides, neutral mucopolysaccharide and protein-bound Ca^{2+} were demonstrated in the droplets. The alkaline phosphatase activity in the enterocyte cytoplasm surrounding the protein droplets indicated the absorption of colostral protein.

The protein droplets in the enterocyte body contained immunoglobulins, first of all IgG molecules, unevenly distributed in individual droplets. We attribute to an uneven distribution the changes in the staining properties demonstrated by means of polychrome dye.

During the preparation of blocks for electron microscopy, we were aware of placing organ pieces to face the blind end of the capsule, i.e. the surface of the block. Glancing over semithin sections from these blocks, we were able to choose the areas where microvilli of enterocytes are lying opposite to each other. Thin sections from these areas are suitable for studying the uptake into enterocytes of colostral milk enclosed by microvilli (Figs 15 and 16).

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HEAT-STABLE SOMATIC ANTIGENS OF A GROUP OF UNCLASSIFIED FLUORESCENT PSEUDOMONADS (UFP)*

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(i) Isolates belonging to a group of unclassified fluorescent pseudomonads (UFP) are similar to *Pseudomonas aeruginosa* not only in cultural behaviour but also in basic serological properties of their somatic antigens. Living bacteria and cultures subjected to prolonged heating at 100 °C or above agglutinated readily in homologous O serum, whereas after exposure to 60 °C, ethanol, saturated sodium chloride and formalin the cells of both species became practically inagglutinable. Surface factors inhibiting the agglutination of living bacteria in O sera being absent, the slide technique was chosen as a routine method for the serological grouping of UFP. (ii) The O antigens of UFP were different in serological specificity from those of *P. aeruginosa*; the two organisms were related only by minor "common" antigens detectable with bacteria heated at 130 °C. One hundred and ten out of 115 UFP isolates were classified into 17 serological groups; O groups 2, 5, 10, 12 and 16 were each further divided into two subgroups. Five isolates reacted in several sera or were unstable. (iii) Serological grouping of UFP is reproducible and adequate for the tracing of isolates and may be helpful for a rapid differentiation of the organism from *P. aeruginosa*.

Fluorescent pseudomonads resembling *Pseudomonas aeruginosa* in growth temperature but distinct from it in several other characteristics were described first in 1972 by HOADLEY and AJELLO [1]. The organisms did not produce pyocyanin, failed to use D-gluconate and D-mannitol as carbon sources and to utilize acetamide as sole carbon and nitrogen source, and to reduce nitrate to nitrogen gas. They were susceptible to kanamycin, were not agglutinated by *P. aeruginosa* antisera and showed little or no pyocin activity. The bacteria, designated UFP (unidentified fluorescent pseudomonas) strains, were classified on the basis of morphological and cultural characteristics into four biotypes [2, 3].

In view of their high degree of biochemical homogeneity, biotype I and III strains are considered to constitute a single species closely resembling *P. aeruginosa* in cultural, biochemical and basic serological properties. UFP strains composing biotypes II and IV appear to be more diverse and until a greater number of strains are available for study, can not be analysed for antigenic structure.

* Presented partly at the Third International Symposium on Pseudomonas, Poznań, Poland, October 7–8, 1977

Materials and methods

Bacterial strains. Sixty-six UFP biotype I and 49 UFP biotype III strains, 7 UFP biotype II and 16 UFP biotype IV strains isolated from surface waters, streams receiving hospital wastes, swimming pools and clinical sources were described by HOADLEY *et al.* [1-3].

To study serological relationships, the following *P. aeruginosa* cultures representing all known O antigens of the species were used: strains 170001-170023 for O antigenic groups and subgroups of LÁNYI's schema [4, 5], strains 1-12 of the HABS schema [6], strain 1-M1 of VERDER and EVANS [7], strain II of SANDVIK [8], strain X of MEITERT [9] and strain Ar 122 of LÁNYI and LANTOS [10].

Immune sera were prepared in rabbits as described for *P. aeruginosa* [4]. For each strain selected as O antigen type culture, at least two different batches of serum were prepared with suspensions heated at 100 °C for 2 1/2 hr and at 75 °C for 1 hr. The sera were preserved with 0.5% phenol or with 50% glycerol + 0.25% phenol.

Slide agglutination was made with 18-20 hr ox-blood agar cultures in appropriate test dilution of O serum [4].

Tube agglutination tests were performed with bacteria harvested from nutrient agar, suspended in physiological saline and treated in the following manner. (a) Living bacteria: the suspensions and serum dilutions contained kanamycin (10 µg/ml) to prevent multiplication during incubation. (b) Bacteria heated at 60 °C for 1 hr, at 100 °C for 1 hr, and at 100 °C for 2 1/2 hr. (c) Bacteria heated at 100 °C for 2 1/2 hr in physiological saline, centrifuged, suspended in small amounts of glycerol, heated at 130 °C for 1 hr and finally resuspended in physiological saline. (d) Bacteria incubated in a solution containing equal volumes of ethanol and physiological saline at 37 °C for 2 days, centrifuged, washed and resuspended in physiological saline. (e) Bacteria incubated in saturated aqueous sodium chloride solution at 37 °C for 2 days, centrifuged, washed and resuspended in physiological saline. (f) Bacteria incubated with 0.5% formalin at 37 °C for 2 days. For details of preparation and adjusting the density of antigens see reference [4].

Absorption of sera was performed with bacteria heated at 100 °C for 2 1/2 hr by the usual technique [4].

Results

1. Serological properties of UFP somatic antigens

Bacterial suspensions exposed to heat and chemical agents were prepared from UFP strains DM-2, DM-41, 325, 204 and 215. The suspensions were tested for agglutination in the homologous sera prepared with antigens heated at 100 °C for 2 1/2 hr.

All strains behaved similarly; an example of the titres obtained with the various kinds of suspensions is presented in Table I. Living cells agglutinated at high titre after incubation at 37 °C. A coarse granular clumping of cells appeared soon after 2 hr. If bacterial multiplication was inhibited by the addition of kanamycin, the titre usually increased on further incubation, and the aggregated bacterial cells of many strains adhered firmly to the walls of tubes, especially at higher (1 : 320-1 : 1280) serum dilutions.

Heating at 60 °C for 1 hr rendered the suspension viscid and inagglutinable. Exposure to 100 °C or above caused the disappearance of viscosity and the reappearance of agglutinability, increasing gradually in titre with the time and temperature of heating. The highest titre (about the same as that obtained with living cells) was demonstrated with bacteria boiled for 2 1/2 hr then heated in

Table I

Effects of heating and chemical agents on the agglutinability of UFP in homologous O antiserum

Reciprocal agglutination titres in tubes incubated at 37 °C

Antigen suspension, strain DM-2	Titre in unabsorbed O serum DM-2	
	2 hr reading	24 hr reading
Living bacteria*	2560	5120
Bacteria heated at 60 °C for 1 hr	0	0
Bacteria heated at 100 °C for 1 hr	160	640
Bacteria heated at 100 °C for 2 ½ hr	640	2560
Bacteria heated at 100 °C for 2 ½ hr + at 130 °C for 1 hr	1280	5120
Bacteria incubated in 50% ethanol at 37 °C for 2 days	0	0
Bacteria incubated in saturated NaCl at 37 °C for 2 days	0	20
Bacteria incubated in 0.5% formalin at 37 °C for 2 days	80	160

* To prevent growth, the agglutination test was performed in saline containing kar.amycin (10 µg/ml)

glycerol at 130 °C for 1 hr. Antigens heated at 100 °C or above showed a coarse granular agglutination in the homologous serum, and adherence of the cells to the tube walls did not occur. The agglutination titre of heated cultures usually increased two-fold when the tubes were incubated in water bath at 50 °C instead of 37 °C.

Treatment of the cultures with ethanol and with saturated sodium chloride caused an almost complete loss of the ability to agglutinate in homologous antisera, whereas exposure to 0.5% formalin usually resulted in a low titre agglutination. If suspensions treated with ethanol, saturated sodium chloride or formalin were subsequently heated at 100 °C for 2 ½ hr, their agglutinability was restored and the titres were similar to those obtained with bacteria heated without a previous exposure to chemical agents.

Mild heating and chemicals influenced only the agglutinability of the cells; immunogenicity and agglutinin-binding capacity remained unchanged.

2. Classification of UFP biotype I and III strains by heat-stable antigens

(a) *Heat-stable "common" antigens.* UFP strains representing individual serogroups cross-agglutinated almost without exception with one another at low titres (1 : 20–1 : 160) if high titre antisera and bacteria heated at both 100 °C and 130 °C were used. Heterologous cross-reaction, appearing as a fine granular clumping of cells, was not observed with living bacteria or with cultures heated at 100 °C only. The cross-agglutinations, attributed to "common"

antigens present in all strains, failed to appear after absorption of the sera with heterologous cultures. The cross-reacting antibodies could be removed not only by UFP, but also by certain *P. aeruginosa* strains. In fact, *P. aeruginosa* strain 170001 (serogroup O1 Lányi = O3 Habs) proved better for this purpose than several UFP strains tested.

(b) *Serogroup-specific heat-stable antigens.* The O antigenic schema for UFP was established on the basis of cross-agglutination tests using both heated and living antigens. Titres obtained by the tube agglutination technique and type strains for the serogroups and subgroups are presented in Table II.

UFP biotype I and III strains were classified into 17 serological groups. Cross-absorption experiments with strains belonging to the same O groups revealed the presence of different major partial antigens in groups O2, O5, O10, O12 and O16. These groups were, accordingly, divided into subgroups.

As seen in Table II, some low titre inter-serogroup reactions were recorded in sera from which antibodies to "common" antigens had been removed by absorption with *P. aeruginosa* 170001; these reactions (e.g. O2 versus O3 and O7 versus O8) were attributed to minor relationships of group-specific antigens.

Living bacteria were cross-tested by the slide method using unabsorbed sera diluted so as to give ++++ reactions with 24 hr blood agar cultures of the homologous strain. The degree of routine slide test dilution of individual sera corresponded well to the titre shown with tube agglutination and living bacteria gave the same pattern of subgroup cross-reactions as did heated ones. There was no evidence of the presence of surface factors inhibiting the O agglutination of living cultures.

In the experiments, sera prepared with bacteria heated at 100 °C for 2 1/2 hr were used as a standard set. After establishing the antigenic schema, bacteria heated at 75 °C for 1 hr were also used for immunization. As compared to those made with boiled antigens, many sera prepared in this manner exhibited a considerably higher titre and gave nonspecific agglutinations less frequently. Antigen O3 (strain DM-41) was an exception: three different batches of serum prepared with 75 °C antigen agglutinated several other O group strains, whereas those prepared with 100 °C antigen reacted with O3 strains only.

3. Antigenic stability of the isolates

Some UFP strains may, as may *P. aeruginosa*, split off multiply agglutinable forms. These cultures are characterized partly by a decreased stability upon heating, partly by reacting in several, but not in all, O sera. If tested by slide agglutination, living blood agar cultures of such strains show a flaky-type aggregation in certain sera, very similar to that described for multiply agglutinable *P. aeruginosa* cultures [11]. Many of these UFP strains showed a characteristic O agglutination in one serum in addition to the flaky-type reaction in

Table II

Classification of UFP biotype I and III strains by group-specific heat-stable antigens
 Reciprocal agglutination titres in tubes at 50 °C for 20 hr with bacteria heated at 100 °C for 2 1/2 hr + at 130 °C for 1 hr

O antigen	Titre in homologous O serum		Titre in other UFP O sera absorbed by <i>P. aeruginosa</i> 170001	Reference strain	Designation	
	Unabsorbed	Abs. by <i>P. aeruginosa</i> 170001			Original*	HNCMB**
1	5120	5120	—	Surface water Georgia, U.S.A.	I 213	172001
2a, 2b	5120	5120	O1 80; O2a, 2c 320; O3 80	Stream receiving hospital wastes, Georgia, U.S.A.	III DM-2	172002
2a, 2c	2560	1280	O2a, 2b 160	Surface water, Belem, Brazil	I 310	172003
3	5120	5120	O2 80	Stream receiving hospital wastes, Georgia, U.S.A.	III DM-41	172004
4	5120	1280	—	Surface water, Belem, Brazil	I 303	172005
5a, 5b	5120	5120	O5a, 5c 2560	Swimming pool, Gainesville, Florida, U.S.A.	I 325	172006
5a, 5c	5120	5120	O5a, 5b 5120	Infected external ear of swimmer, Gainesville, Florida, U.S.A.	I 330	172007
6	5120	1280	—	Swimming pool Ontario, Canada	I 423	172008
7	5120	5120	O8 40	Surface water, Belem, Brazil	I 307	172009
8	5120	2560	O7 40	Hospital waste, Georgia, U.S.A.	I 204	172010
9	2560	1280	—	Surface water, Georgia, U.S.A.	I 222	172011
10a, 10b	5120	5120	O3 20; O10a, 10c 2560	Stream receiving hospital wastes, Georgia, U.S.A.	III DM-17	172012
10a, 10c	5120	5120	O10a, 10b 160	Surface water, Belem, Brazil	I 320	172013
11	5120	5120	O2 20	Stream receiving hospital wastes, Georgia, U.S.A.	III DM-20	172014
12a	5120	5120	O12a, 12b 640	Stream receiving hospital wastes, Georgia, U.S.A.	I DM-49	172015
12a, 12b	2560	640	O12a 5120	Hospital waste, Georgia, U.S.A.	I 207	172016
13	5120	2560	—	Surface water, Georgia, U.S.A.	I 215	172017
14	5120	5120	—	Surface water, Belem, Brazil	I 304	172018
15	5120	5120	—	Hospital waste, Georgia, U.S.A.	III 206	172019
16a, 16b	5120	5120	O16a, 16c 5120	Surface water, Georgia, U.S.A.	I 220	172020
16a, 16c	5120	2560	O16a, 16b 1280	Surface water, Belem, Brazil	I 308	172021
17	2560	2560	—	Surface water, Belem, Brazil	I 316	172022

* Hoadley and Ajello; Roman numerals indicate the biotype

** HNCMB = Hungarian National Collection of Medical Bacteria, National Institute of Hygiene, Budapest, Hungary

other sera; these cultures were regarded as "antigenically degraded" members of the corresponding O group. Four multiply agglutinable strains failed to give a ++++ reaction in any of the O sera and one strain showed spontaneous agglutination in saline (see figures in brackets in Table III). The appearance of multiple agglutinability was observed in both kinds of sera used (i.e. those prepared with bacteria heated at 75 °C and at 100 °C).

To check their stability, UFP isolates chosen as type strains for the antigenic groups and subgroups were subjected to serial subculture on nutrient agar plates at 37 °C by starting on each day from single colonies. Although in some cultures more than one colonial form appeared, with the exception of one strain, the cultures remained antigenically stable after 30 subcultures. Strain DM-20 (O11) tended to split off multiple agglutinable forms.

4. Antigenic relationship between UFP and *P. aeruginosa*

To test the relationship between "common" antigens of the two species, heated suspensions (100 °C 2 1/2 hr + 130 °C 1 hr) were prepared from all UFP type strains and from eight *P. aeruginosa* type strains (O1, O2, O3a, 3b, O4a, 4d, O5a, 5b, 5c, O6, O7a, 7b and O10a of LÁNYI's schema) and examined by cross-agglutination in tubes. Although finely granular cross-agglutinations occurred between UFP and *P. aeruginosa*, they were less regular and lower in titre as compared to intraspecies cross-reactions.

As "common" factors remain masked if living cultures are used, the relationship between the O antigens of the two species was tested by slide agglutination of type strains representing all known O antigens. These examinations revealed no significant relationship between group-specific antigens of UFP and *P. aeruginosa*.

5. Routine method of serological grouping of UFP

For routine typing of UFP isolates, slide agglutination is the method of choice. By the usual technique applying living blood agar cultures grown at 37 °C overnight and O sera in routine test dilution, the group antigens can be determined as reliably as by the tube technique. Since sera for the slide method must be free from flagellar and fimbrial antibodies, heated antigens have to be used for immunization. Sera prepared with antigens heated at 75 °C may be recommended; they are usually higher in titre and, with some exceptions, more O antigen specific than sera made with boiled bacteria.

Pooled sera were prepared by mixing appropriate amounts of individual O sera and diluting the pool so as to give ++++ slide reaction with strains belonging to the corresponding serogroups. Aspecific cross-reactions in carefully checked pooled sera are infrequent and usually occur only with multiply agglutinable strains. The following O sera were pooled:

I: 1, 2, 3

II: 4, 5a, 5b, 5a, 5c, 6

III: 7, 8, 9, 10a, 10b, 10a, 10c, 11

IV: 12a, 12b, 13, 14, 15, 16a, 16b, 16a, 16c

For the determination of O groups, the sera can be used unabsorbed; only the absorption of serum O7 with antigen O8 and *vice versa* may be necessary. For subgroup determination, the sera should be absorbed with the appropriate antigen as evident from the formulae shown in Table II.

Table III

Distribution of UFP biotype I and III isolates according to serogroups and sources

O antigen	Biotype	Number of isolates						Total
		Man	Surface water, Georgia	Wastes and streams receiving hospital wastes, Georgia	Surface water, Belem, Brazil	Swimming pool, Gainesville, Florida	Swimming pool, Ontario, Canada	
1	I	—	2	—	—	—	—	2
	III	1	2 (1)	—	—	—	—	3 (1)
2a, 2b	I	—	5	1	—	—	—	6
	III	—	—	7	—	—	—	7
2a, 2c	I	—	—	—	2	—	—	2
3	III	—	—	23	—	—	—	23
4	I	—	—	—	2	—	—	2
5a, 5b	I	—	1 (1)	—	—	14 (1)	—	15 (2)
	III	—	1	—	—	—	—	1
5a, 5c	I	1	—	—	1 (1)	—	—	2 (1)
6	I	1	—	—	—	—	1	2
7	I	—	1 (1)	—	1	—	—	2 (1)
8	I	1	—	1	1	—	—	3
9	I	—	1	—	—	—	—	1
10a, 10b	I	—	—	—	4	—	—	4
	III	—	—	3 (1)	—	—	—	3 (1)
10a, 10c	I	—	—	—	1	—	—	1
11	III	—	—	2	—	—	—	2
12a	I	—	—	1	—	—	—	1
12a, 12b	I	—	—	4 (1)	—	—	—	4 (1)
	III	—	—	4	—	—	—	4
13	I	—	5	—	—	—	—	5
	III	—	—	3	—	—	—	3
14	I	—	—	—	2	—	—	2
15	I	—	1	1	2 (1)	—	—	4 (1)
	III	—	—	1	—	—	—	1
16a, 16b	I	—	1	—	—	—	—	1
16a, 16c	I	—	—	—	3	—	—	3
17	I	—	—	—	1	—	—	1
Untypable	I	—	2 (2)	—	1 (1)	—	—	3 (3)
	III	—	—	1 (1)	—	—	—	1 (1)
Unstable	I	—	—	1 (1)	—	—	—	1 (1)
Total		4	22 (5)	53 (4)	21 (3)	14 (1)	1	115 (13)

Non-bracketed figures: total number of isolates

Bracketed figures: isolates agglutinating in more than one serum or unstable cultures

6. Serogroup distribution of UFP isolates

As shown in Table III, seven out of the twenty serological units were encountered in two or more different geographic areas. It is remarkable that in serogroups and subgroups O1, O2a, 2b, O5a, 5b, O10a, 10b, O12a, 12b, O13 and O15 both biotype I and III strains were represented. However, biotype I and III strains were identical in all respects save patterns of flagellation which differed among recent isolates only; when maintained on laboratory media, the morphological difference was lost.

7. Preliminary studies on heat-stable somatic antigens of UFP biotype II and IV strains

To examine the properties of UFP II and IV biotypes, suspensions of strains II 219, II 231, IV 230 and IV 236 were heated at different temperatures and exposed to ethanol, saturated sodium chloride and formalin as described for UFP biotype I and III. The cultures behaved similarly: living bacteria agglutinated at high titre, mild heat and the chemical agents caused inagglutinability, whereas suspensions heated at 100 °C or above again became agglutinable.

UFP biotype II and IV strains contained "common" antigens related to those of *P. aeruginosa* and UFP biotype I and III. With respect to O antigens, UFP II and IV isolates were classified into 6 provisional serogroups.

Some O group specific serological relationships of UFP II and IV strains to *P. aeruginosa* and UFP I and III were as follows. Strains IV 237 and IV 238 reacted at considerable titre in UFP I serum O1. UFP strain II 231, UFP I O12a, 12b and *P. aeruginosa* O10a were interrelated by minor somatic antigens. A more definite relationship was recorded between UFP strain IV 233 and *P. aeruginosa* O3a, 3d.

Discussion

The heat-stable somatic antigens of UFP isolates are very similar in basic serological properties to *P. aeruginosa* O antigens. Earlier studies [4] have shown that *P. aeruginosa* cells (i) contain, in addition to serogroup-specific O antigens, heat-stable "common" antigens responsible for low-titre intra-species cross-reactions; (ii) possess no thermolabile somatic antigens inhibiting the agglutination of living cultures in O antiserum; (iii) become inagglutinable after heating at 50–80 °C or treatment with certain chemical agents; (iv) are restored to agglutinability after heating at 100 °C or above; (v) retain their immunogenicity and agglutinin-binding capacity after exposure to heat and chemical agents. In the present study the same properties were shown to be characteristic of UFP strains.

Antigens causing the low-titre cross-agglutination of heated suspensions of UFP are similar in behaviour to "common" antigens described in *P. aeruginosa*. In serological specificity these factors are not identical in the two species. This is shown by the fact that, although "common" antibodies to UFP can be absorbed with certain *P. aeruginosa* strains, detectable inter-species reactions are less frequent than intra-species ones and, if present, are lower in titre.

Serogroup-specific somatic factors (O antigens) are also similar in nature in the two species: they can be determined by the use of living cultures or of suspensions heated at high temperature and neither *P. aeruginosa* nor UFP contains heat-labile surface factors inhibiting O agglutination.

In serological specificity there is, however, a sharp difference between *P. aeruginosa* and UFP O antigens. UFP isolates can be distinguished by serological examination not only from typical *P. aeruginosa* strains, but also from pyocyanin negative strains, which are characterized by antigens identical with those present in pyocyanin-producing cultures [11].

Inagglutinability of *P. aeruginosa* cells exposed to mild heat and chemical agents has been attributed to a swelling or an alteration in the structure of an unidentified substance present on the cell surface. The elimination by heating at high temperature of the masking effect on O agglutinability has been associated with the destruction of the unknown substance [4]. The phenomenon of inagglutinability in UFP may be attributed to the same mechanism.

Another serological similarity between *P. aeruginosa* and UFP is the occurrence of isolates showing flaky-type slide agglutination in several O antisera. Multiply agglutinable *P. aeruginosa* cultures have been shown to occur in nature and have been obtained *in vitro* as an effect of phage action [10].

The present findings indicate that UFP biotype I and III isolates represent a group of organisms similar to *P. aeruginosa* not only in cultural but also in basic serological properties. It is also obvious that, on the basis of important biochemical differences and of an entirely different specificity of O antigens, the two organisms should be separated taxonomically and the group constituting UFP biotypes I and III deserves the rank of an individual species.

As many isolates belonging to the two biotypes of the UFP group are antigenically identical or closely related, it is justified to include them in the same antigenic schema.

From the results of the present study it may be concluded that the UFP group can be divided into antigenic groups and subgroups sufficient in number to use serological examination for the epidemiological tracing of isolates. The difference in serogroup specificity between *P. aeruginosa* and UFP may be helpful as an aid in the rapid differentiation of the two groups of organisms.

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EFFECT OF BORDETELLA PERTUSSIS VACCINE ON THE COURSE OF LYMPHOCYTIC CHORIOMENINGITIS (LCM) VIRUS INFECTION IN SUCKLING MICE PRETREATED WITH DIANHYDRODULCITOL (DAD)

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In earlier experiments *Bordetella pertussis* vaccine was found to enhance and dianhydrodulcitol (DAD) to weaken cellular immune response to intracerebral LCM virus infection in suckling mice. *B. pertussis* vaccine proved to inhibit the restrictive effect of DAD produced on the immune response in mice when 2- to 4-days-old animals were pretreated with DAD and subsequently, at the age of 16 to 18 days of life, treated with *B. pertussis* vaccine then infected with LCM virus. Consequently, *B. pertussis* vaccine enhanced the immune response previously affected by DAD.

A cellular immune reaction is the basis of the lethal lymphocytic choriomeningitis due to intracerebral (i. cer.) LCM virus infection. Consequently, the course of the infection depends on the animal's immune status [1]. Earlier experiments showed that the course of i. cer. LCM virus infection changed when the animals were subjected to *Bordetella pertussis* vaccine treatment and virus infection at the age of 2 weeks and also when the animals were pretreated with DAD at the age of 2 to 4 days and infected with LCM virus at the age of 2 weeks. *B. pertussis* vaccine facilitated and DAD inhibited the development of meningitis following LCM virus infection; this means that *B. pertussis* vaccine amplified and DAD restricted the cellular immune response [2, 3]. In the present experiments the effect of *B. pertussis* vaccine treatment on the course of LCM virus infection has been studied in mice pretreated with DAD at the age of 2–4 days.

Materials and methods

Experimental animals. CFLP mice of both sexes were used.

Dianhydrodulcitol treatment. Dianhydrodulcitol, the epoxide of mitolactol (DBD) is an alkylating cytostatic agent. The substance was kindly supplied by the Chinoin Chemical and Pharmaceutical Works Ltd, Budapest. The material was dissolved in sterile distilled water and used within 30 min. Experimental animals were treated with 5 mg/kg of the drug intraperitoneally (i.p.) on a single occasion.

B. pertussis. *B. pertussis* vaccine contained 30×10^9 /ml killed bacteria suspended in physiological saline (Human Institute for Serobacteriological Production and Research, Budapest). The animals were injected with 0.3 ml *B. pertussis* vaccine i.p. on two occasions, the vaccine contained a total of 18×10^9 killed bacteria for each animal.

LCM virus infection. The W.E. strain maintained at our Institute in serial mouse brain passages and stored at -70°C was used. PBS (phosphate buffered saline pH 7.0) was used for

the preparation of the brain suspensions and virus dilutions. Virus titration was performed in adult 6 to 8 weeks old mice, by inoculating the animals i.cer. with a pretitrated 100 LD₅₀ quantity in 0.03 ml of the virus. The development of neurological symptoms characteristic of lymphocytic choriomeningitis (tremor, convulsions) were controlled twice daily and deaths were registered.

Recovery of virus. At the end of the experiment 1:10 dilutions of brain suspensions were prepared from each of the surviving animals and mouse groups were inoculated with the suspension i.cer. Presence of the virus could be confirmed on the basis of the characteristic neurological symptoms and of deaths in the inoculated mouse groups.

Lymph organs. (a) Body weight and the spleen weight of the sacrificed animals in the various groups were determined separately to calculate the relative spleen weight.

$$\text{Mean relative spleen weight} = \frac{\text{mean spleen weight, mg}}{\text{mean body weight, g}}$$

(b) The quotient of mean relative spleen weight in the individual experimental groups and of mean relative spleen weight in the untreated control groups gave the spleen index.

Statistical evaluation of data. Mean relative spleen weight and its standard error was calculated. Significant differences between the experimental and control values were evaluated by the Student *t* test; the level of significance was $p = 0.05$.

Experimental procedure. The experiments were carried out on 220 mice belonging to 26 litters. The number of animals was reduced to 8 to 10 per litter. Intraperitoneal DAD treatment was given to 6 to 8 mice at 2 to 4 days of age in 18 litters. The remaining mice of the same litters were given PBS solution in an identical way. On the 13th and 14th day after DAD treatment half of the DAD treated animals was inoculated i.p. with *B. pertussis* vaccine, the other half received PBS solution in an identical way. On the day of the second *B. pertussis* vaccine treatment, mice from 9 litters were infected with LCM virus i.cer. Of these animals the DAD treated mice constituted the DAD-LCM group, those subjected to DAD and *B. pertussis* vaccine treatment made up the DAD-P-LCM group and animals treated with PBS the C-LCM group. Mice in 9 litters not infected with the virus were inoculated with normal mouse brain suspension i.cer. Of these animals the DAD treated mice formed the DAD groups, the DAD and *B. pertussis* vaccine treated animals the DAD-P, and those given PBS solution the C group.

The effect of *B. pertussis* vaccine on the lymph organs of the animals and on the course of the LCM virus infection was studied in mice from an additional 8 litters. Animals in these litters were given PBS solution at the age of 2 to 4 days. Three-quarters of the animals received

Table I
Mouse groups and treatments

Group	No. of mice	i.p. treatment at the age of		i.cer. inoculation at the age of 16-18 days
		2-4 days	16-18 days	
DAD-LCM	29	DAD	PBS	LCM virus
DAD-P-LCM	25	DAD	<i>B. pertussis</i>	LCM virus
C-LCM	18	PBS	PBS	LCM virus
P-LCM	32	PBS	<i>B. pertussis</i>	LCM virus
DAD	30	DAD	PBS	×
DAD-P	30	DAD	<i>B. pertussis</i>	×
C	24	PBS	PBS	×
P	16	PBS	<i>B. pertussis</i>	×
C _a	16	PBS	PBS	×

× normal brain suspension

B. pertussis vaccine 13 and 14 days after treatment and the remaining siblings received PBS solution. On the day of second *B. pertussis* treatment, $\frac{2}{3}$ of the *B. pertussis* vaccine treated animals were infected with LCM virus and the remaining ones were given normal mouse brain suspension. *B. pertussis* vaccine treated and virus infected animals constituted the P-LCM group. Of the animals inoculated with normal brain suspension, those treated with *B. pertussis* vaccine belonged to the P group and those treated with PBS solution to the C_a group. Table I demonstrates the mouse groups, the number of mice in the individual groups and the treatment applied.

Experiments were terminated on the 21st day after virus inoculation. The rate and time course of deaths following virus infection are presented in Fig. 1.

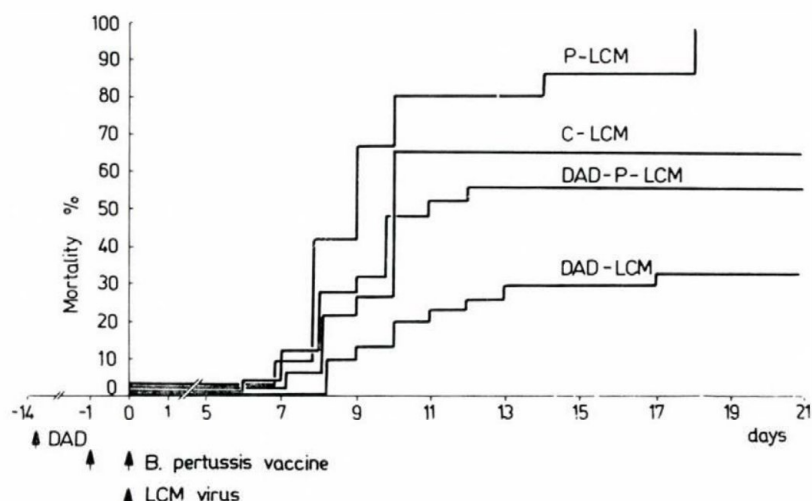


Fig. 1. Time course of mortality in LCM virus infected mouse groups

Results

Sixty-six per cent of the animals in the not pretreated (C-LCM) group died between the 7th and 10th day following virus infection. Only 33% of the DAD pretreated and virus infected (DAD-LCM group) animals died; death occurred between the 8th and 17th day after infection. Mortality rate was thus lower in the DAD-LCM group than among their siblings not pretreated with DAD (C-LCM group). Animals which had received DAD treatment previous to *B. pertussis* vaccine treatment and were infected with the virus (DAD-P-LCM group) exhibited a higher mortality rate (56%) than the animals not having *B. pertussis* vaccine treatment (DAD-LCM group) and their mortality rate approached the rate observed in the group subjected only to virus infection (C-LCM group). Hundred per cent of the animals which received *B. pertussis* vaccine treatment as well as virus but no DAD pretreatment (P-LCM group) died between 7 to 18 days following virus infection. Neither disease nor death occurred after i.cer. inoculation in the groups not infected with virus. Virus was reisolated from each of the animals surviving the infection, which

proved that these animals had become virus carriers. To study the effect of the treatments on the lymphoid system, 12 animals from each of the groups not subjected to virus infection were sacrificed on the 10th day after i.cer. inoculation, and the relative spleen weights and spleen indexes were determined. Table II shows the results.

Table II
Mean relative spleen weight and spleen index

Group	Mean relative spleen weight	Significance	Spleen index
P	9.9 ± 1.8	$p < 0.001$	1.6
C _a	6.2 ± 1.8		1.0
DAD	4.9 ± 1.0	$p < 0.02$	0.8
C	6.0 ± 0.9		1.0
DAD-P	7.3 ± 2.3	$p > 0.05$	1.2

The data in Table II indicate that the mean relative spleen weight and spleen index were lower in the DAD group and higher in the P group than in the untreated control group. Post mortem examination of the animals showed that the DAD treated mice developed spleen atrophy whereas *B. pertussis* vaccine treatment caused spleen hypertrophy. In the DAD and *B. pertussis* treated animals spleen atrophy or hypertrophy could be observed at the time when the majority of the LCM virus infected animals were already dead. Mean relative spleen weight in the DAD-P group was higher than in the DAD and C groups, but the difference was significant only in relation to the DAD group values (DAD-P vs. DAD: $p < 0.01$, spleen index: 1.4). Thus *B. pertussis* vaccine produced a hypertrophic effect also in the DAD treated animals. In mice treated simultaneously with DAD and *B. pertussis* vaccine neither spleen atrophy nor hypertrophy could be observed as compared to the controls.

Discussion

Mortality rate was higher in the *B. pertussis* vaccine treated and virus infected (P-LCM) group and lower in the DAD treated and virus infected (DAD-LCM) group than in the untreated and virus infected (C-LCM) group. These results agree with earlier data [2, 3] according to which *B. pertussis* vaccine treatment enhanced and DAD treatment inhibited the outcome of LCM virus infection in the form of lethal meningitis. After DAD treatment, *B. pertussis* vaccine treated and virus infected (DAD-P-LCM) group animals reacted to the infection in a similar fashion as their untreated littermates (C-LCM group).

The inhibitory effect of DAD on the cellular immune response could not be detected in the DAD-P-LCM group since the *B. pertussis* vaccine stimulated the immune response in DAD treated mice, too. In earlier experiments it was found that DAD and *B. pertussis* vaccine produce antagonistic effects on the lymphoid system in mice: while DAD treatment caused spleen atrophy, *B. pertussis* vaccine induced spleen hypertrophy [2, 3]. The previous and the present results agree well concerning the effects of the two drugs on the lymphoid organs and *B. pertussis* vaccine had a hypertrophizing effect on DAD treated mice (DAD-P group). Relative spleen weight and cellular immune responses showed a direct correlation in DAD and *B. pertussis* vaccine treatment. Relative spleen weights lower than normal were associated with a decreased immune response, and higher than normal spleen weight with an increased immune reaction. After combined treatment, the relative spleen weights were nearer to the control values and the immune response was comparable to that of the control animals.

As to the clinical importance of the results, it might be interesting that pertussis vaccine eliminated the inhibitory effect of DAD treatment on the immune response of mice.

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STIMULATION OF THE CELLULAR IMMUNE RESPONSE TO LYMPHOCYTIC CHORIOMENINGITIS (LCM) VIRUS INFECTION IN SUCKLING MICE

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Death occurred earlier and its rate was higher in one-week-old mice treated with phytohaemagglutinin (PHA) and subsequently inoculated intracerebrally with LCM virus than in their virus infected but untreated littermates. Thus PHA treatment contributed to the outcome of LCM virus infection in the form of lethal meningitis. The course of LCM virus infection in 1-week-old PHA treated mice was similar as in the untreated 2-week-old mice. This indicates that PHA treatment accelerated the development of cell mediated immunological capacity in suckling mice.

The basis of the lethal lymphocytic choriomeningitis induced by intracerebral (i.cer.) LCM virus infection is a cellular immune reaction and thus its course depends on the immunological competence of the animals, consequently on their age as well. The infection causes lethal choriomeningitis in adult mice with intact immune system. Meningitis does not develop in newborn mice, the animals survive the infection and become virus carriers. In suckling mice the rate and time of death depend on the age of the animals at the time of infection [1, 2].

Earlier experiments revealed that the meningitis due to LCM infection developed earlier and the mortality rate was higher in mice treated with PHA than in the untreated animals. This suggested that PHA treatment enhanced the cellular immune reaction to LCM virus infection [3].

In the present experiment the effect of PHA treatment on the course of LCM virus infection has been studied in suckling mice.

Materials and methods

Experimental animals. CFLP mice of both sexes were used.

Phytohaemagglutinin treatment was performed with Famitogen (The Wellcome Research Laboratories). For a single intravenous treatment the LD₅₀ of the drug was found to be 50 mg/kg for 18-day-old mice. A single 100 mg/kg dose given intraperitoneally failed to have a toxic or lethal effect on 11-day-old mice.

LCM virus. The applied W. E. strain was maintained in serial mouse brain passages. PBS (phosphate buffered saline solution pH 7.0) was used for the preparation of brain suspension and dilutions. Virus titration was performed by the i.cer. inoculation of young adult mice, using the pretitrated 100 LD₅₀ dose of the virus. Development of the neurological symptoms characteristic of lymphocytic choriomeningitis (tremor, convulsion) and the death of the animals were controlled twice every day.

Recovery of virus. Mouse groups were i.cer. inoculated with 1 : 10 dilution of the brain suspensions prepared separately from each of the surviving animals. Recovery of LCM virus was determined by the characteristic neurological symptoms and deaths.

Histological examinations. Brain sections of the animals succumbing during the experiment as well as of those sacrificed on the 10th day were stained with haematoxylin-eosin.

Results

To study the course of LCM virus infection in mice of different ages a preliminary experiment was undertaken (Experiment I). One, 2 and 6 weeks old mice were inoculated i.cer. with the same dose (100 LD₅₀) of LCM virus. Control animals were given uninfected mouse brain suspension. Each litter included 8 to 10 mice. The experiment was terminated 28 days after the infection. Mouse groups and treatment are presented in Table I.

Table I
Mouse groups and their treatment in Experiment I

Group	Number	Age of mice	i.cer. treatment
LCM-I	50	1 week	LCM virus
C-I	16	1 week	×
LCM-II	50	2 weeks	LCM virus
C-II	16	2 weeks	×
LCM-III	16	6 weeks	LCM virus
C-III	16	6 weeks	×

× uninfected mouse brain suspension

Rate and time course of deaths during Experiment I are presented in Fig. 1.

Of the adult mice (LCM-III group) 100% died in 6 to 8 days after the infection, with symptoms characteristic of lymphocytic choriomeningitis. Of the 2-week-old animals (LCM-II group) 52% died in 7 to 18 days after the infection. Of the 1-week-old mice (LCM-I group), 18% died in 10 to 19 days. Virus was recovered from the brain of each animal surviving the 28th day subsequent to virus infection, thus these animals were virus carriers. No death occurred in the uninfected control groups. Four animals from each of these groups (C-I, C-II and C-III) were sacrificed 10 days after inoculation for histological study.

Considering the result of the preliminary experiment, the effect of PHA treatment on LCM virus infection was studied in groups containing 1-week-old mice (Experiment II). Fourteen litters were used, the number of mice in

each litter being reduced to 8. Half of the animals in each litter was treated i.p. with 100 mg/kg PHA on three subsequent days and the other half was given PBS solution in an identical way. On the last day of the treatment mice of 10 litters were inoculated with 100 LD₅₀ of LCM virus. PHA pretreated and virus infected mice formed the PHA-LCM group and those given PBS solution and infected with virus made up the C-LCM group. Simultaneously with the infection, mice of 4 litters were inoculated with uninfected mouse brain suspension. Of these animals, those treated with PHA formed the PHA groups, and those given PBS solution constituted group C. The various groups of mice used in the experiment as well as the types of treatment are presented in Table II.

Table II
Groups of mice and their treatment in Experiment II

Group	Number	i.p. treatment	i.cer. inoculation
PHA-LCM	40	PHA	LCM virus
C-LCM	40	PBS	LCM virus
PHA	16	PHA	×
C	16	PBS	×

× uninfected mouse brain suspension

Experiments were terminated on the 21st day after infection. Four animals were sacrificed from the C and PHA groups 10 days after inoculation for histological examination. Fig. 2 summarizes the rate and time course of deaths in Experiment II.

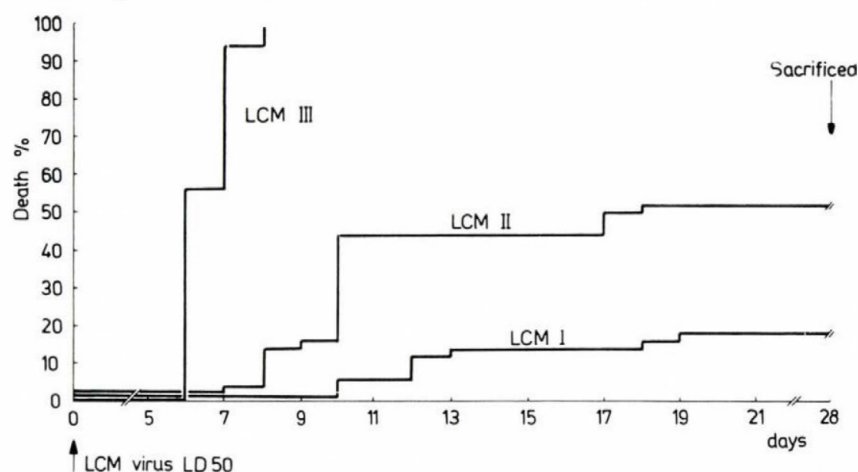


Fig. 1. Time course of the mortality rate in Experiment I

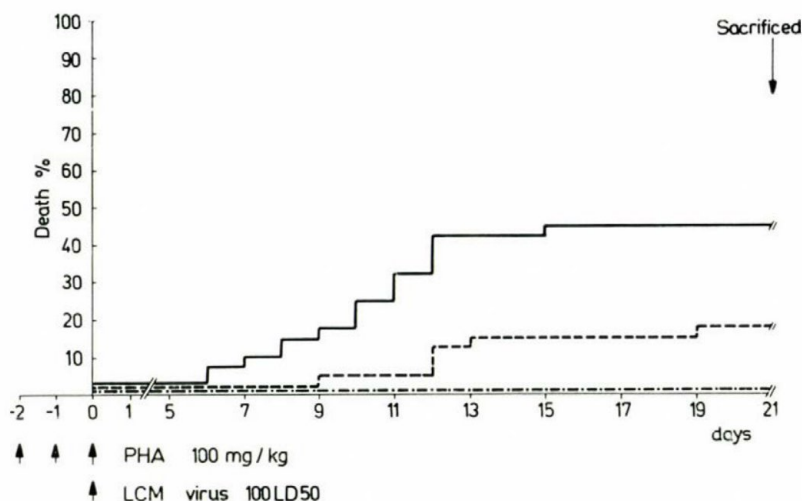


Fig. 2. Time course of the mortality rate in Experiment II. — PHA-LCM; - - - C-LCM; - . - . - PHA

Eighteen per cent of the animals in the virus infected but not PHA treated group (C-LCM group) died between the 10th and 19th day. Forty-five per cent of the virus infected animals treated with PHA (PHA-LCM group) died between the 5th and 15th day. Thus, the mortality rate was higher and deaths occurred earlier in the PHA treated and virus infected group than in the untreated littermates. All mice surviving the 21st day became virus carriers as shown by the successful recovery of LCM virus from their brain. Neither disease nor death occurred in the uninfected C and PHA groups.

Results of histological examination of the brains are presented in Table III.

Table III
Histological examination of the brains

Experiment	Group	No. of brains studied				
		Total	degree of lymphocytic infiltration on the leptomeninges			
			+++	++	+	-
I	LCM-III	16	16 (100%)	—	—	—
	LCM-II	24	3 (12%)	9 (38%)	12 (50%)	—
	LCM-I	8	—	2 (25%)	6 (75%)	—
II	PHA-LCM	12	1 (8%)	4 (33%)	7 (59%)	—
	C-LCM	7	—	2 (28%)	5 (72%)	—

Lymphocytic infiltration was present in the brain of LCM infected mice in each case, showing different degrees of infiltration in the individual groups. Severe lymphocytic infiltration was found in each of the 6-week-old mice (LCM-III group) in Experiment I. In the group of 2-week-old mice, only 12% of the animals showed a severe, 38% a moderate and 50% a slight infiltration. No severe infiltration was found in the 1-week-old mice, where 25% displayed moderate and 75% a slight infiltration (LCM-I group). Thus, the degree of lymphocytic infiltration of the leptomeninx depended on the age of the animals at the time of infection. The degree of infiltration increased parallel with age.

In Experiment II, severe leptomeningeal infiltration was found in 8%, moderate infiltration in 33% and a slight one in 59% of the PHA treated one-week-old mice. In untreated one-week-old mice (C-LCM group) there was no severe infiltration; in 28% of the animals it was moderate and in 72% slight. The degree of the lymphocytic infiltration was thus more marked in the virus infected and PHA treated group (PHA-LCM group) than in animals not subjected to treatment (C-LCM group). No lymphocytic infiltration appeared in the mice inoculated with normal brain suspension (C-I, C-II, C-III, C and PHA groups).

Discussion

T lymphocytes play a major role in the development of lymphocytic choriomeningitis following i.cer. LCM virus infection. The disease and death are due to the cytotoxic activity of the LCM virus antigen-specific T lymphocytes against cells expressing viral antigens in the choroid plexus, meninges and ependyma [4-6]. In the various animal species the immune system is developed in various degrees at birth. The appearance of T cells in the lymph organs and the development of their immunological competence are a continuous event which begins after birth and progresses for several weeks. Cells capable of recognizing cellular surface antigens foreign to the cell's "proper" antigen, were studied by the GvH reaction and in mixed lymphocyte cultures. The results showed that these cells began to appear 1 to 2 weeks after birth and were not present in the spleen of newborn mice [7, 8]. The presence of cells with specific cytotoxicity against LCM virus infected cells has been studied in cell suspensions obtained on the 9th day after i.p. LCM virus inoculation from the spleen of mice immunized at different ages. No such cells could be demonstrated in the spleen of mice immunized neonatally. The cytotoxic activity of the spleen cell-suspension increased parallel with age and its rate in the 3rd week of life was half that of adult mice [9].

The results of preliminary experiments (Experiment I) in agreement with data in the literature [10] have shown that the death rate after i.cer.

LCM infection depends on the age of the infected animal. The number of survivors decreases from neonatal to adult age; old mice were found to succumb earlier than younger ones. Histological examination, too, showed that the lymphocytic infiltration of the leptomeninx was more extensive in old than in young mice. These data are in good agreement with general knowledge of the immune pathology of LCM virus infection [4-6] as well as of the extrauterine development of cell-mediated immunity of mice [7-9]. Thus, there is a direct relationship between the development of immunological capacity and the occurrence of lethal meningitis due to i.cer. LCM infection.

The results of Experiment II concerning the effect of PHA treatment on the course of LCM virus infection in one-week-old mice showed that PHA treated and virus infected animals died earlier and displayed a higher mortality rate (PHA-LCM group) than their untreated littermates (C-LCM group). These results agreed with the histological findings of the dead animals in that the leptomeningeal infiltration in the PHA-LCM group was more marked than in the untreated C-LCM group.

This suggests that in suckling mice PHA treatment contributes to the outcome of LCM infection in the form of lethal meningitis. The present results confirm those obtained earlier in experiments performed on adult mice [3, 11].

Comparing the course of LCM virus infection in one-week-old PHA treated animals of Experiment II (PHA-LCM group) with that of untreated two-week-old mice in Experiment I (Figs 1 and 2) as well as the histological findings of the dead mice in these groups (Table III, groups PHA-LCM and LCM-II), PHA treated one-week-old mice were found to react to LCM virus infection similarly as the 2-week-old untreated animals. This indicates that PHA treatment had accelerated the development of cell mediated immunological capacity in suckling mice.

The stimulating effect of PHA treatment on lymphocyte transformation and mitosis may explain the observed stimulation of the cellular immune reaction to LCM virus infection. PHA is known to affect the T lymphocytes both *in vitro* [12, 13] and *in vivo* [14]. Considering the significance of T lymphocytes in the development of meningitis as the consequence of i.cer. LCM virus infection, it may be assumed that PHA accelerates the development of cell mediated immunity in suckling mice by stimulating the proliferation of T lymphocytes.

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ENDOTOXIN TOLERANCE IN RATS TREATED WITH ANTILYMPHOCYTE SERUM

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Rats were treated with rabbit anti-rat lymphocyte serum (ALS). The subsequent selective immunosuppressive effect on the immune response of thymus dependent antigen was shown by immunization with sheep red blood cells (SRBC). Endotoxin tolerance could be evoked in ALS treated animals. This suggests that the establishment of endotoxin tolerance is independent of thymus function and makes it possible to enhance the nonspecific resistance of immunosuppressed patients with a transplanted organ.

Bacterial lipopolysaccharide (LPS) exerts a variety of biological effects in mammals [1]. An increase in nonspecific resistance after endotoxin treatment is believed to be related to the induction of endotoxin tolerance [2–5]. The reticuloendothelial system (RES) and the participation of some type of immune response appear to be involved in the onset of tolerance. The absence of nonspecific resistance in immunosuppressed patients is a major problem of transplantation procedures. It is well known that the majority of transplanted patients die of common septicaemia because their ability to mount an immune response is suppressed. Antilymphocyte serum is a potent immunosuppressant commonly used in organ transplantation. For this reason the possibility of augmenting a patient's nonspecific resistance after ALS treatment has a great importance.

LPS can influence the immune system by acting as a mitogen for B lymphocytes [6, 7] and interacts with T cells [8, 9], especially since it seems to act by a T cell independent antigen [10–13]. In the experiments to be reported, induction of endotoxin tolerance in animals treated with ALS is described. The results revealed the independence of endotoxin tolerance from thymic functions.

Materials and methods

Wistar rats of both sexes weighing 100 g were used. ALS was prepared in rabbits by injecting 2×10^8 rat thymus lymphocytes intravenously on days 1 and 14. The animals were bled on day 21. ALS and normal rabbit serum (NRS) were inactivated and absorbed with rat red blood cells. The cytotoxic activity of ALS was tested by the dye exclusion test and by chromium release using ^{51}Cr labelled rat thymocytes. The selected sera were cytotoxic at a dilution above 1 : 200. Rats were injected intraperitoneally with 2 ml of ALS or NRS/100 g body weight 3 and 1 days before immunization and induction of tolerance, respectively. Details of the immunization procedures, the plaque forming assay and antibody titration have been reported

[14, 15, 16]. Endotoxin tolerance was evoked by administering 100 μ g radiodetoxified endotoxin [17] and on the next day the animals were challenged with a lethal dose (5 mg) of *Escherichia coli* O89 LPS.

Results and discussion

As can be seen in Table I, ALS treatment had an immunosuppressive effect on the antibody response to SRBC. The decrease was statistically significant when compared to the results obtained with NRS or in the untreated group. The titres were practically the same as those of the background level [16]. In agreement with the data of other authors, antibody response to a thymus dependent antigen (SRBC) was suppressed in ALS treated animals.

Table I

Immunosuppressive effect of ALS on the immune response to sheep red blood cells

Treatment (days of treatment)	Immunizing antigen	Splenic PFC		Log2 of reciprocal serum titre	
		direct	indirect	MES + MER	MER
none*	SRBC 4×10^8 i.v.	n.d.	n.d.	8.8 ± 0.3	3.2 ± 0.4
NRS (-3; -1) i.p.	SRBC 4×10^8	2350 ± 250	4700 ± 100	7.0	2.6
ALS (-3; -1) i.p.	i.v.	680 ± 100	290 ± 110	2.6**,***	0**,***

ALS = antilymphocyte serum

NRS = normal rabbit serum

SRBC = sheep red blood cells

PFC = plaque-forming cells

MES = 2-mercaptoethanol sensitive

MER = mercaptoethanol resistant

* each group consisted of 5-10 rats. Splenic PFC and antibody titres were determined 5 days after immunization

** statistical significance in untreated group, $p = 0.01$

*** statistical significance in NRS treated group, $p = 0.05$

Whether ALS treatment interfered with the induction of tolerance to endotoxin was investigated by using radiodetoxified endotoxin [17]. Thus, ^{60}Co -gamma-ray detoxified LPS was given to rats to see if it increased resistance to a lethal dose of LPS. Results are shown in Table II. It is evident that in spite of the suppression of thymus function with ALS, induction of tolerance to endotoxin was normal. Our data provided no information on how the tolerance developed but it was obviously independent of ALS sensitive thymic cells.

Based on these results we assume that natural (nonspecific) resistance to infection can be enhanced in animals immunosuppressed by ALS. The finding may offer some advantage (protection against common septicaemia) in organ-transplanted immunosuppressed patients.

Table II

Effect of ALS treatment on the induction of tolerance to radiodetoxified endotoxin

Group*	Pretreatment 72 hr – 24 hr	Tolerance induction	Challenge at 24 hr	Mortality dead/total
1	—	—	LPS 5 mg i.v.	18/20
2	—	Radiodetoxified endo- toxin 100 µg i.v.	LPS 5 mg i.v.	0/20
3	ALS	Radiodetoxified endo- toxin 100 µg i.v.	LPS 5 mg i.v.	0/20

* each group consisted of 20 rats

ALS = antilymphocyte serum

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HEXAGONAL CRYSTALLINE ARRAYS OF ADENOVIRUS TYPE 1 HEXONS

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Separated, highly purified and concentrated adenovirus type 1 soluble hexon capsomers were crystallized by dialysis against 0.5 M acetate buffer. The crystallization process was followed electron microscopically. In the early phase of the crystallization, groups of a few hexons began to appear, then the two-dimensional crystal lattices grew gradually to a size of 1–2 μm . Simultaneously three-dimensional crystals of tetrahedral and prismatic shapes developed. The hexons in the two-dimensional crystal lattice formed regular dense arrays corresponding to the hexagonal packing. Analysis of the crystal structure revealed 15–20% local irregularity (short range disorder) and about 10% deviation in the values of the lattice constant if determined from three different directions. The average lattice constant values showed considerable differences in different preparations. Angles formed by non-parallel hexon rows deviated by a few degrees from the regular hexagonal order. Consequently, the position of the hexons in dense two-dimensional crystals was found slightly skew and irregular, although each unit stayed within a certain distance as compared to its equilibrium position defined theoretically in the network. Dislocations were frequently found to disturb the regular arrays. The extra hexon row developing between two rows diverted them from their original direction. At these sites the crystal lattice slanted and the dense array of the hexons loosened. High resolution electron microscopy revealed fine linking structures between the hexons. In several cases the aggregated hexons failed to show a ring-like appearance, they were situated in lying — profile — position and the hexon-building polypeptide fibres became visible. The diameters of the hexons and the distance between them were measured in three directions and the size of the hexon-building polypeptides was determined as well.

The protein envelop of the adenovirus — the capsid — consists of 252 morphological units, the capsomers. Twelve of these, the vertex capsomers are called pentons, together with their projection (called fibre) and the remaining 240 are the hexons [1]. Hexon capsomers can be extracted and purified from adenovirus infected cells. Highly purified and concentrated hexon preparations were successfully crystallized by some research groups including our team [2–7]. In the course of this procedure, three-dimensional crystals of tetrahedral and prismatic shape developed. Before and during the development of the three-dimensional crystals, two-dimensional hexon crystalline arrays were detected; their structure differed from the holey two-dimensional hexon crystals [8–14]. Hexons in the two-dimensional crystal sheets were situated in one layer and displayed the same array as those on the surface of the virion. This similarity is of a special significance for the detailed study of the structure of the two-dimensional hexon crystals, i.e. for the study of the intermolecular forces cohering the hexons. These investigations may lead to

the recognition of the factors influencing the assembly of the viral capsid, the maturation of the infective virion.

Materials and methods

Separation and purification of the soluble hexon. After disintegrating the adenovirus type 1 infected HEp-2 cells, the soluble protein components were separated from the virion by ultracentrifugation (MSE Superspeed 50) [11]. From the fraction containing the capsid proteins and situated above the virion band in the centrifuge tube, the hexon, penton and fibre were separated after dialysis in 0.01 M phosphate buffer (pH 7.0) by anion exchange chromatography on DEAE Sephadex A-50 column. Phosphate buffer with a NaCl content increasing by 0.025 M was used as eluent. Hexon containing fractions were subjected to repeated chromatography and gel filtration as described earlier [12]. The NaCl content of the eluent was eliminated by dialysis against 0.01 M phosphate buffer. Subsequently the protein concentration was determined by the method of LOWRY *et al.* [13]. Extinction values were measured in a Pye Unicam SP 800 spectrophotometer.

Crystallization of the hexon capsomers. Highly purified hexon preparations were concentrated and dialysed against 0.5 M acetate buffer (pH 4.5), at 4 °C for 72 hr without stirring. The materials were placed in glass tubes and stored at 4 °C. The slightly opalescent supersaturated protein solution was studied by light microscopy and with a JEM 100 B electron microscope at 60 kV, after uranyl acetate staining. From the 2nd to the 3rd day of storage at 4 °C, aggregation of the hexon capsomers became apparent by electron microscopy in contrast with the untreated preparation which failed to display such regular array even after several months of storage. At the same time, crystals of tetrahedral and prismatic shape were detected by light microscopy [7].

Results

Hexagonal packing. The crystallization process was followed by electron microscopy after dialysis against acetate buffer. In the early phase of the crystallization, two-dimensional hexon groups consisting of 10 to 100 capsomers varying in size began to appear. During the process of crystallization the crystal lattices reached 1–2 μm in diameter. In the two-dimensional crystal sheets the hexons were situated in regular dense rows corresponding to the hexagonal packing.

To characterize the structure of the crystal lattice, the theoretically best "equilibrium" positions of the individual hexons in the crystal lattice as well as the angles formed by the three non-parallel hexon rows were determined. The lattice constant was determined in the same three directions. To this end, electron micrographs showing the most regular arrays were chosen and the centre of each hexon was marked. These centres were reproduced on photographs and were connected corresponding to the hexagonal packing with three non-parallel lines, according to the principles given in Fig. 1. The points of intersection given by the three lines showed the theoretically correct position of the individual capsomers within the lattice. The displacement of the dots marking the centres of the hexons indicated the local irregularity, i.e. the "short range disorder" of the crystal lattice [14]. Several hexons were shifted

from the centre (Fig. 1/b). In certain cases the rate of this displacement reached even 15–20% of the given row distance in the lattice. Thus the “short range disorder”, i.e. the local irregularity was considerable. The average centre to centre spacing, i.e. the lattice constant (l.c.) which was determined on the

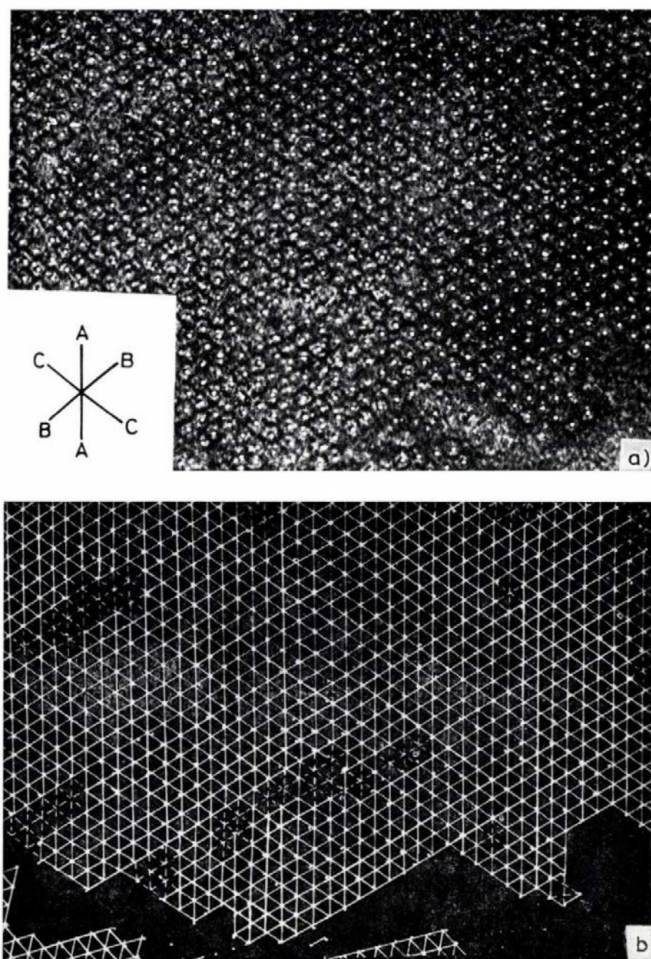


Fig. 1. (a) Electron micrograph of a dense two-dimensional hexon crystal ($\times 295\,200$). Staining with uranyl acetate. Centres of the hexons are marked by white dots. Directions of the three non-parallel hexon rows are presented in the left lower corner of the microphotograph. (b) Dots marking the centres are connected network-like, corresponding to the crystal part presented in Fig. 1/a ($\times 296\,600$). Dotted line indicates the part of the network where the hexon centres could not be determined in the original microphotograph. Straight line segments follow closely the rows of dots as far as their displacement showed an irregular distribution on both sides of the line. Three non-parallel lines were drawn to intersect each other in one common point of the network. These points define the equilibrium position of the capsomers in the lattice. The displacement of the dots marking the hexon centres, indicate the rate of the short range disorder in the network. Comparison of the distance between the parallel lines over large areas illustrates the long range disorder

basis of several thousand measurements, varied considerably in the different preparations. The average l.c. in Fig. 1 was $122.8 \text{ \AA}$ and that in Fig. 2 $111 \text{ \AA}$. Significant differences were found in the l.c. values of the same preparation if they were determined from the three different non-parallel directions. The direction of the three non-parallel hexon rows is presented in the left lower corner of Fig. 1. Determining the average values in the hexon rows of A — A direction the l.c. was $130.5 \pm 2.6 \text{ \AA}$, that in the hexon rows of B — B direction: $116.4 \pm 1.8 \text{ \AA}$ and in the C — C direction $121.6 \pm 1.7 \text{ \AA}$. In Fig. 2 in the rows of A — A direction the l.c. was $117 \pm 4.8 \text{ \AA}$, in the B — B rows: $105.4 \pm 4.5 \text{ \AA}$ and in the C — C direction: $110.2 \pm 2.9 \text{ \AA}$. Approximately 10% was the difference between the highest and lowest average l.c. in the hexon rows of the three different directions. According to the l.c. values in the lattice, the long

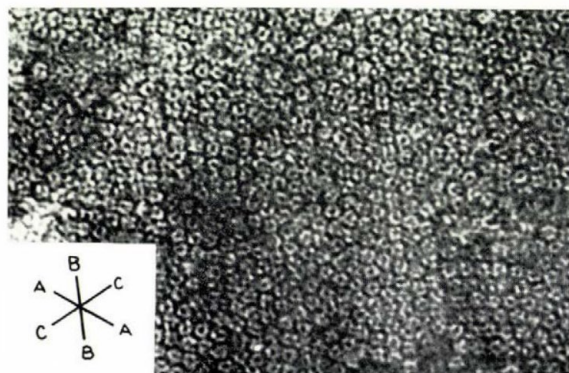


Fig. 2. Electron micrograph of a part of a two-dimensional hexon crystal. Uranyl acetate staining. The diagram in the left lower corner displays the direction of the hexon rows

range disorder — the distance between the parallel rows in Fig. 1 — showed similarly an about 10% deviation in the hexon rows of three different directions. Consequently, the long range disorder was slighter than the short range disorder. Figure 3 demonstrates the l.c. values measured in the hexon rows of three different directions in a two-dimensional crystal presented in Fig. 1. The angles formed by the non-parallel rows of the lattice deviated with 1–5 degrees from the 60° and 120° angles of the regular hexagonal network, taking any two direction as the basis. Thus, the hexon capsomers in the dense two-dimensional lattice, showed a slightly skew and irregular hexagonal packing.

Dislocations. Dislocations disturbing the regularity could be observed on several occasions. The extra hexon row inserting between two hexon rows diverted them from the initial direction around the point of origin. At these sites the crystal lattice slanted considerably and the dense array of the hexons has loosened up. They were present in a limited number in a given area, consequently the hexagonal array disappeared at these sites. The irregularly inserted

new hexon row proceeded up to one side of the crystal lattice whereafter the hexagonal packing developed again. Insertion of islet-like dislocations was detected also in certain cases which failed to continue in either direction of the extra row. "Complementary" dislocations also appeared in opposite directions on both sides of the regular hexon row bending slightly in S form. Formation of two dislocation rows enclosing an approximately 60° angle was frequently detected at the point of origin of the dislocations, in the network. The dislocations appeared with different frequency in different preparations. Certain preparation contained no or only few (Fig. 1) dislocated hexon rows, whereas other crystal lattices were filled with them (Fig. 4). The appearance of these latter crystal-line arrays was looser and less regular. Bent hexon rows could easily be detected in them.

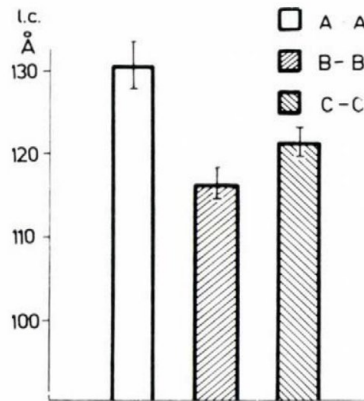


Fig. 3. Average lattice constant values measured in three non-parallel hexon rows of the two-dimensional crystal, presented in Fig. 1. Significance: A - A versus B - B: $p < 0.001$; B - B versus C - C: $0.01 > p > 0.001$; A - A versus C - C: $p < 0.001$. Statistical analysis of the data was performed with Student's t test. Level of significance, $p = 0.05$.

Hexons, inter-hexonal distances and linking units. According to the three directions of the hexon rows in the crystal lattice, the diameter of the hexons was measured individually. The average distance between the hexons was calculated as well by subtracting the total of the hexon diameters from the total of the l.c. values of the hexon rows of identical direction. The average diameter of the hexons in the A - A rows in the two-dimensional lattice presented in Fig. 1 was 95 ± 1 Å and the average distance 23.3 ± 5.3 Å. In the rows of B - B direction the average diameter was 93.6 ± 0.8 Å and the average distance 20.4 ± 1.3 Å. In the C - C direction these values were 93.2 ± 3.1 Å and 28.9 ± 3.3 Å, respectively. For comparison, values obtained in Fig. 2 are presented in the following: direction A - A average diameter 91.7 ± 3.9 Å, average distance 27.5 ± 4.4 Å; direction B - B average diameter 92.8 ± 6.3 Å, average distance 17.3 ± 2.1 Å; and direction C - C average diameter 92.1 ± 2.2 Å and average distance 15 ± 2.7 Å.

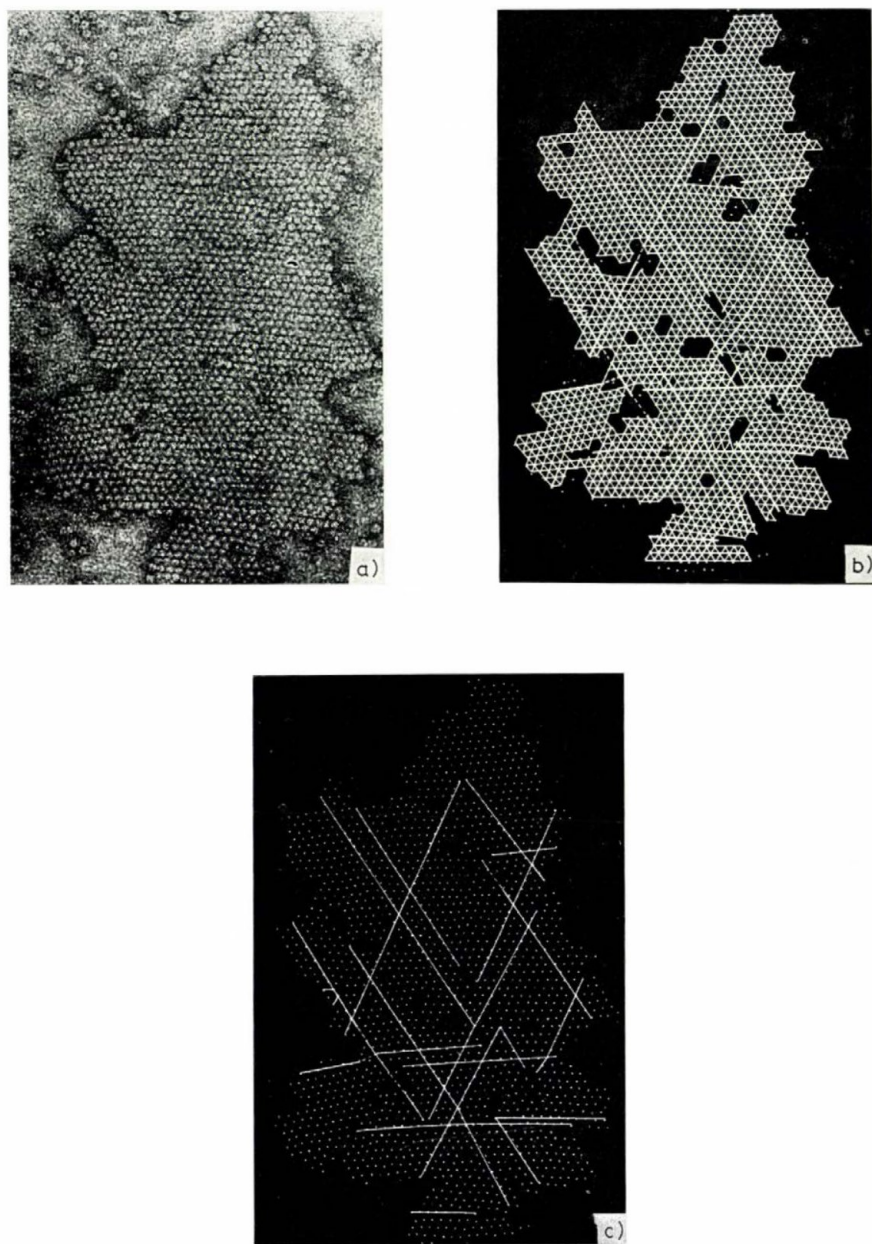


Fig. 4. (a) Electron micrograph of a dense two-dimensional hexon crystal ($\times 149\,000$). Uranyl acetate staining. Hexon centres are marked. (b) Centre marking dots are connected network-like according to the principles given in Fig. 1. Lines are curved according to the bends of the rows of dots and terminated at the sites where the dot rows stop owing to dislocations or to other factors. All these sites the network was left empty. Thick lines indicate the lines of the dislocated hexon rows in the network. (c) Dots indicating the hexon centres and straight lines underlining the dislocated hexon rows in the network

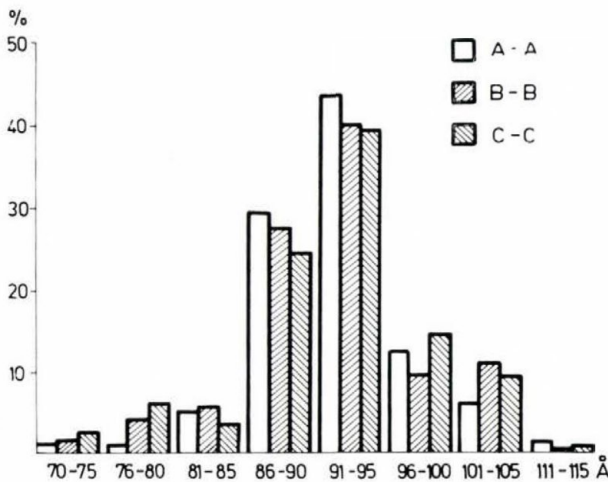


Fig. 5. Frequency distribution of hexon diameter measured in three non-parallel hexon rows

Figure 5 demonstrates the frequency distribution of the hexon diameters. The largest diameter was 115 Å. In our studies only one giant hexon was detected with a diameter surpassing 130 Å in all the three directions (Fig. 6). It was surrounded by 9 neighbouring capsomers and a dislocated row started from one side. Thus the disorder due to the large size had been equilibrated. The diameter of the hexons in the rows of three different directions deviated only slightly. The average distance between them also showed differences. These failed to be parallel with the l.c. differences in hexon rows of different directions, i.e. with the deviations of the long range disorders.

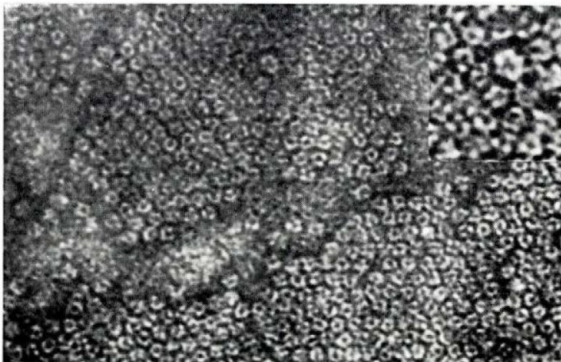


Fig. 6. Electron micrograph of an unusually large hexon. The difference in size is well visible as compared with other hexons in the two-dimensional lattice. The diameter reaches or even surpasses 130 Å in each direction. It is surrounded by 9 neighbouring hexons and a dislocated hexon row starts from one of its sides ($\times 300\,000$). Magnification of insert $\times 500\,000$

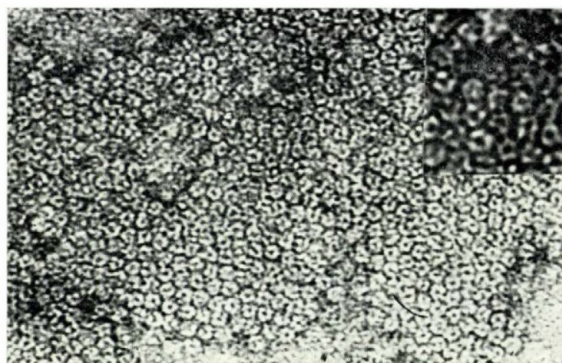


Fig. 7. Electron micrograph of a hexagonal crystalline array ($\times 300\,000$). Fine projections linking each hexon with its six neighbours are well discernible. They appear occasionally in pairs. Insert magnification $\times 500\,000$

In high resolution electron micrographs, slender structural elements connecting the hexons in the lattice were observed, frequently as projections connecting all the six neighbours (Fig. 7). In some cases two projections connected two neighbouring capsomers. Projections ranged between 5 and 15 Å in diameter and between 20 and 35 Å in length. In several lattices the usual ring-shaped form of the hexons failed to be recognizable in smaller or larger areas which instead displayed a filamentous network structure. The size of the filaments varied between $25\text{--}35 \times 110\text{--}130$ Å, but deviations from these values were often observed. Determination of their size was difficult owing to their dense array and superposition. Randomly two filaments ran parallel; they appeared to be connected at one of the ends and at their middle part. At these points they displayed a slight swelling. The filaments of the network are most likely polypeptides which build the hexons and become visible by the hexons lying in



Fig. 8. Electron micrograph of hexons in profile. Ring-like hexons are also visible in the filamentous network ($\times 300\,000$). The filaments are probably the structural polypeptides of the hexons in lateral position. Two neighbouring filaments frequently run approximately parallel

lateral, profile position. Such solitary "lying" hexons could be seen also in crystal lattices consisting of the usual ring-like formations. On the other hand, solitary ring-like hexons could be observed in larger filamentous areas.

These filamentous network areas in the two-dimensional crystal lattice or between two separate crystal lattices give the impression of being their continuation. Whether there was some regularity in this structure has not been found out. It is difficult to clarify the problem as the linking projections are most likely also included in the filamentous network.

Discussion

Detailed structure analysis of the two-dimensional crystalline arrays revealed that certain hexons were significantly displaced from their theoretically correct position, which refers to a considerable local (short range) disorder. The lattice constant shows also considerable deviations, though less in different areas of the same lattice. Significant differences were found within the same areas in the average values determined in the directions of the three non-parallel hexon rows. Similar differences were found in the l.c. values of the six neighbouring hexons. Angles formed by non-parallel hexon rows also deviated by a few degrees from the 60° and 120° angles.

Leaving out of consideration the crystal defects due to dislocations and other factors, slightly skew and irregular hexagonal packing characterizes the structure of the two-dimensional crystals. Nevertheless each unit stays within a certain determined distance as compared to its equilibrium position determined theoretically in the lattice. This order characterizes the structure and refers to the existence of a specific interaction among the hexons which maintains the sixfold coordination. Underfocussed high resolution electron microphotographs showed that each hexon was linked with its six nearest neighbours by fine projections. These linking structures must be flexible and elastic and the individual hexons can change their position in the lattice as far as the elasticity of their own connecting structures as well as the position of their neighbouring hexons — defined similarly by the linking units — allow it. This might explain the somewhat smaller size of the connecting structures found in our experiments as compared with those determined by HORNE *et al.* [15] in studies of adenovirus type 5. The hexon diameters in the lattice were similar to the data of other authors [1, 16], but if measured in three different directions, the length of the diameters of the same hexons was slightly deviating.

The linking elements may provide means to determine which end of the hexons — the one facing the inside of the virion or the opposite one — is visible on the electron micrograph. Hexons are assumably connected on their basis, i.e. on their end facing the inside of the virion. Thus on microphotographs

where linking structures are visible, the hexons show their inner end facing the core. It is not, however, certain that each hexon in the crystal lattice is oriented identically. An assembly of hexons of various orientations is also feasible. This may provide the explanation of the phenomenon that linking structures are hardly, or not at all, visible in certain areas. Hexons in the lateral, profile position appear occasionally in crystal lattices consisting of upright ring-like formations, while ring-shaped hexons in upright position (Fig. 8) may be found among the hexon-building polypeptide chains in the filamentous network aggregation. Connecting structures must also play a role in these cases. To determine the kind of interactions required for the maintenance of such regular structures displaying at the same time several disorders, more information is needed concerning the intermolecular forces. The orientation of the upright hexons to each other has to be determined according to the three hexon-building polypeptides, and the relationship of the threefold symmetry corresponding to the three polypeptides and the hexagonal packing requires elucidation.

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MAMMALIAN ONCORNAVIRUSES: AN INTRODUCTORY REVIEW*

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Mammalian oncornaviruses have now been discovered in a wide variety of species from various mammalian classes such as the rodents, primates and artiodactyla. At present three main categories of these viruses are known. They are usually classified as viruses of the B, C and D type. While viruses of the B type are confined to rodents and D type viruses to primates, viruses of the C type have been demonstrated in all three classes as well as in species of yet other mammalian groups. Although the C type viruses differ widely in their biological effects and in their biochemical and immunological makeup, it has recently been stressed that they all may be derived from one common ancestor virus [1]. Such studies on e.g. immunological similarities between the C type viruses of different mammals are usually focused on the endogenous rather than the exogenous viruses. Nucleic acid information of endogenous viruses is represented in the species in which the virus is found, while this is not the case for the exogenous viruses, which may come from a completely different class of mammals.

Oncornaviruses cause tumours under a number of circumstances and the capacity to induce malignancies has been studied mainly in rodents. Inbred mice are the most important tools to study the susceptibility of the host to oncogenic viruses of the B and C type. In fact, the first mammalian oncornavirus was isolated from mice and demonstrated to cause mammary cancer [2], while the second was also found in mice and shown to cause thymic lymphoma [3]. These findings were the result of the creation by geneticists of inbred strains with high incidences of cancer.

The discoveries of the type B and C viruses by BITTNER and GROSS, respectively, was followed by isolations of a number of highly oncogenic agents in the mouse, such as the Rauscher, Friend, and Moloney isolates, all causing malignancies in a short time. With such isolates virologists were able to eluci-

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date the structural, biochemical and immunological characteristics of the oncornaviruses and the geneticists could show the genetic complexity involved in the susceptibility to the virus-induced neoplastic diseases. In the fifties these "exogenous" viruses were the most important tools. However, in the sixties and seventies the question arose how such highly oncogenic agents could have been generated from the often "harmless" endogenous oncornaviruses. Molecular biological studies revealed the wide-spread occurrence of not only one, but at least three classes of endogenous C type viruses in the mouse and obviously many of the isolated "endogenous" viruses were not oncogenic at all. The xenotropic C type viruses of the mouse were the classical example of non-oncogenic agents, while the ecotropic viruses in inbred mice and the amphotropic viruses of wild mice were thought to be the oncogenic representatives.

Still, the original isolates causing "rapid" haematological disorders and the sarcomas, were not really "endogenous" viruses. Part of their genomes was represented in the chromosomes, but part did not seem to be represented. How could they have been generated? What was the difference between these variants of C type viruses selected for oncogenic capacity and the "endogenous" viruses with both ecotropic and xenotropic host range? The answer to this question came from the AKR mouse strain known for its high leukaemia incidence, in which the first murine C type virus was found by GROSS about 30 years ago.

In the late sixties it was shown that the expression of that virus and its group-specific antigen was under host genetic control [4-7]. While two genes controlled the expression of XC + virus, about 4-6 genes controlled that of MuLV-gs. In segregating crosses between AKR and low leukaemia strains, there was a good correlation between XC+ virus expression and leukaemogenesis and the involvement of the endogenous viral genomes in leukaemogenesis was shown definitely in partially congenic NIH-SWISS mice with the so-called Akv genes. However, the extremely high and early leukaemia incidence of the AKR strain was not found in the congenic strains with genetic susceptible background strains [for review, see 8]. Apparently the isolation of individual Akv genes lead to a low or moderate incidence of leukaemia and additional genes were required for the high incidence in AKR. In fact, FURTH, who created the AKR strain, suggested as early as in the forties that many genes were involved in leukaemogenesis of the strain [9].

Epigenetic studies on the expression of MuLV and its antigens in the AKR strain by various groups [10, 11], that is the study of the relation between differentiation and virus expression, revealed a surprisingly low content of virus in the target organ for leukaemogenesis, the thymus. Only in the pre-leukaemic age of about 6-8 months did virus titres increase sharply in this organ, suggesting that an infectious process preceded the development of leukaemia. Careful analysis of the raise in the thymus of virus titres showed that not

only the exotropic but also the xenotropic MuLV levels had increased [12]. Until then, xenotropic virus had not been implicated in the disease of this mouse strain.

In a classical study it was then shown by HARTLEY *et al.* [13] that the virus variants found in AKR lymphomas, as well as lymphomas of the congenic strains with Akv genes, exhibited both ecotropic and xenotropic host ranges and that these variants were not "mixtures" but genetically stable recombinants between the two endogenous viruses. Normal tissues, except preleukaemic thymuses, did not yield recombinant viruses some of which showed transforming capacity on mink lung cells (therefore called mink lung cell focus forming or MCF viruses). Molecular biological studies confirmed that the leukaemias contained recombinants between eco- and xenotropic MuLV's and the "env" region was involved in the recombination event.

These studies implicated that, at least in the AKR system, induction of endogenous viruses without a further "spreading" process, did not lead *per se* to leukaemia. This was in fact already suspected on the basis of the Fv-1 effect [for review, see 8]. Apparently virus induced in very few cells had (1) to be produced by these cells, (2) to "exogenously" infect surrounding cells (in which the endogenous viral information stayed "silent"), and (3) to form certain recombinants with the other class (eco- versus xenotropic or *vice versa*), before transformation could take place. This immediately implicated that it would be possible to vaccinate against AKR leukaemogenesis, although the virus causing the disease was "endogenous", since it had to leave the cell at a certain point before leukaemogenesis started. When this notion developed, the groups of HUEBNER and SCHÄFER started vaccination programs in the AKR and kept mice, from a very early age on, under high levels of antibodies to the viral envelope components (group-specific for eco- and xenotropic MuLV). Both groups succeeded in obtaining AKR mice of well over one year and they were able to reduce or delay leukaemogenesis substantially. Thus a "practical" solution for the cure of this "genetic" disease was found.

However, the practical solution did not follow the complete understanding of leukaemogenesis as it occurs in this leukaemia model, the AKR mouse strain. The precise mechanism of the malignant change from thymocyte to thymic leukaemia cell is not known. It is known that many genetic factors play a critical role in the leukaemogenic process, such as (i) the major histocompatibility complex, (ii) the dominant gene for "spreading" of ecotropic virus, the Fv-1 system (of which more alleles than "n" and "b" are likely to play a role) and (iii) recessive genes interfering with ecotropic MuLV expression as shown with congenic strains.

Mammalian oncornaviruses together with the avian viruses are no doubt the main tools for the study of carcinogenesis of lymphoid tissues and mammary gland tissues [for review, see 14]. Expression of these viruses as a function

of differentiation and dedifferentiation in the host has taught us, much about the malignant process on the one hand and the developmental processes on the other hand. Mammalian oncornaviruses are in the centre of modern biology and will provide us with answers about the precise mechanism of the cancerous disease.

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RECENT TRENDS IN THE STUDY OF ONCORNAVIRUSES*

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In a short introductory remark it is very difficult and, to be more exact, impossible, to dwell in detail on any of the numerous interesting trends in the study of oncornaviruses.

My choice will be subjective. First of all, I would like to point out the great success achieved in understanding the fundamental properties of oncornaviruses: their genome structure, replication cycle peculiarities, biochemical, immunochemical, and morphological principles of structural organization of oncovirus virions. One of the most important achievements in this field is the identification of "sarc" genes of some sarcomatous viruses, a work that promises to deepen and precisely to define our knowledge on the mechanisms of cell transformation by oncornaviruses.

However, doing justice to an outstanding progress made in this field, we cannot pass by in silence some other similarly important fields in the study of oncornaviruses.

Here, first of all, one should dwell on cattle leukaemia virus and feline leukaemia virus. As it is known, a question about the viral nature of cattle leukaemia has been under discussion for a long time, but only during the recent years has this problem been elucidated to such an extent that it has become possible to say with confidence that enzootic leukaemia of cattle is caused by oncornavirus BLV. This virus spreads horizontally. In the majority of cases BLV infection leads to a symptom-free virus-carriership and in a few subjects to leukaemic disease. The knowledge of these phenomena, as well as the availability of methods for BLV infection diagnosis elaborated in recent years, allow to proceed to scientifically well-grounded programmes of eradication of enzootic cattle leukaemia; this would be of great economic use.

Feline leukaemia has no economic significance, but the discovery of its pathogenic agent, feline leukaemia virus (FeLV), the study of its spreading and the elaboration of methods for its identification undoubtedly belong to

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the remarkable achievements in oncovirology during the last decade. By the way, the main possibility of tumour eradication by revealing virus-carriers and preventing their contacts with virus-free animals was shown for the first time just on that very model of feline leukaemia and on a virus that causes "natural" tumour under "natural" conditions.

As you can imagine the branch devoted to primate oncoviruses is the most close to me as a primatologist working in oncovirology. As far as 10 years ago no such viruses were known to us. By now, a great number of them have become known. It is true that in some cases it is difficult to determine the origin of these viruses, but this is a question for special discussion.

Primate oncovirology has discovered a number of new phenomena which are of great interest. Suffice it to note the phenomenon of interspecies transmission of endogenous oncornavirus from one animal species to another for which that virus proves to be oncogenic.

SSV-1 and GALV are the examples of such a transmission occurring in natural conditions. No doubt that deciphering the origin of these viruses belongs to the greatest achievements of oncovirology.

Although the problem of endogenous viruses is not new, it seems that the discovery and thorough investigation of the biology, molecular biology and immunology of primate endogenous oncornaviruses is one of the most significant and constructive trends in this field. When studying the group of viruses, the fundamental problem of the endogenous oncornaviruses which in many respects exceed the limits of proper oncovirology, becomes clearly apparent.

As far as the role in oncogenesis of the endogenous viruses is concerned, there are few data but we have to mention the investigations indicative of the oncogenicity of pseudotypes of the primate endogenous oncoviruses.

We owe to primate oncovirology the discovery of a new group of viruses, the D-type oncornaviruses though many things are not yet clear about these viruses but they are undoubtedly interesting objects of research.

When speaking about primate oncovirology you can not pass over in silence such a vital area as human oncovirology.

Nowadays, there are two points of view in this field. The first one is that the search for completely infectious human oncovirus is hopeless. It only makes sense to look for virus-specific sequences, virus information that can be associated aetiologically and pathologically with tumour origin.

According to the second point of view, infectious oncornaviruses of man do exist and can be determined, though their isolation and particularly their thorough investigation and characterization are difficult and sometimes impossible.

The supporters of each of these points of view have a great number of arguments in store. In my opinion, the development of the second trend seems to be potentially more promising.

It seems necessary to study more comprehensively the possible pathogenicity for human beings of animal oncogenic DNA- and RNA-containing viruses. Among the latter, first of all the BLV, FeLV, AvLV and Marek's disease virus and some other viruses should be investigated.

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VIRUSES AND HUMAN CANCER*

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Seventy-five years ago the French scientist BORREL presented the hypothesis that viruses could cause cancer. In 1908 ELLERMAN and BANG showed that leukosis of the hen could be transmitted by a cell-free extract. Two years later it was shown by PEYTON ROUS in the United States that a transmissible sarcoma of the fowl, the Rous sarcoma, was caused by a virus and from that time emerged the problem we are discussing. It took, however, about 40 years before the idea had been accepted. One of the pioneers in this field who kept the idea alive was FRANCISCO DURAN-REYNALS, who in the *American Journal of Medicine* in 1950 published a paper with the title "Neoplastic infection and cancer" in which he stressed the possibility that also human tumours could be caused by viruses. Today it is interesting to remember that the honorary lecture at the first meeting of the European Tumour Virus Group in London in 1961 was given by PEYTON ROUS. It is impossible to summarize in a few words the work of 70 years in tumour virology and I will therefore in a very simple way try to touch upon some of the problems related to viruses in human cancer.

It has several times been pointed out that there is a clear correlation between occurrence of virus-induced benign tumours like papillomas and fibromas among animals and human beings. When turning to cancer the correlation is not as clear. It is therefore good to remember the view of Sir MACFARLANE BURNET who some years ago stated that virus-induced cancer in animals is seen under very special conditions in very inbred populations and that they could or should be regarded as a laboratory product. This is easy to accept when discussing for instance mice, but when speaking about the avian viruses and viruses in domestic animals like cats, pigs, cattle and dogs, we usually claim that the viruses and the diseases occur under natural conditions. I do think, however, that even here we have to count with a big laboratory experiment, with highly inbred populations.

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Looking then at the different viruses which could be considered, there is I think at the moment a slight decrease in the interest in adenoviruses as causes of human tumours, since tests of 7 out of 31 adenoviruses for homology in cells of two types of human tumours did not show any homologous sequences. This does of course not exclude them as candidates and in any case they are good models for *in vitro* experiments.

Papilloma viruses could be somewhat more promising, and, it is, as a matter of fact, surprising that most people have in discussions neglected the human papilloma viruses. It has, however, been stressed recently that they fulfill quite a number of criteria for a potential carcinoma virus, they are widely spread, several of the viruses have oncogenic potential in experimental models, they induce premalignant lesions which possibly might change into malignancy and it is quite possible also, although less studied that they behave in the similar way as some other known tumour viruses, namely being incorporated in the genome of cells.

Experiments performed in order to find out if sequences homologous to these viruses occur in human carcinoma cells would perhaps be at least as profitable as experiments with the adenoviruses. One urgent task would, however, be to grow these viruses *in vitro*. It has also been claimed that in cases with condyloma there is a higher incidence of malignancies of the uterine cervix and for that reason the combination of the effect of the papilloma virus and the herpes virus should be considered.

Then to the group of herpesviruses. This is the group which still gives most promise, as far as the correlation to human tumours is concerned. There is not any reason to repeat the work which has been done on the EB-virus, and the correlation to Burkits lymphoma and nasopharyngeal carcinoma. I do think that, in addition to all the work which has been done and which has been mentioned and presented at this symposium, the recent work on the propagation of EB-virus in epithelial cells may greatly add to our knowledge about the pathogenesis of nasopharyngeal carcinoma and about EB-virus infection as a whole.

At the moment, however, many have the feeling that the final proof about the relation of EBV and the different types of carcinomas can only be obtained by a vaccination trial in the region where the tumours are prevalent, in other words, repeating the experiments which have been done with the Marek disease virus.

The relationship between herpes simplex virus and carcinoma is not as convincing as with EB-virus and the nasopharyngeal carcinoma or the Burkits lymphoma. The prediction that viral DNA sequences should be present in cancer cells has been fulfilled in tumours associated with EB-virus but herpes simplex type 2 DNA sequences have not reproducibly been demonstrated in cancer cells. A new approach seems to be the *in situ* hybridization by demonstra-

tion of herpes virus messenger RNA in the cytoplasm of cervical cancer cells as done by McDougall who uses 3H labelled HSV-DNA. The multiple etiology seems to be certain as far as EB-viruses are concerned. One may have to consider it also in the case of the carcinoma of the cervix. The condylomas just mentioned might be an additional factor.

In this connection it is probably also worth mentioning that the possible presence of other viruses in the uterine cervix has been overlooked. In a trial which we did in Helsinki when evaluating the rubella virus vaccine, rubella virus was isolated in a high percentage from smears of the uterine cervix. Those cells might thus be susceptible to many virus infections and one should probably explore the possibility of dual infections of the cervix cells considering the occurrence of cancer. Cytomegalo virus is of course also a very interesting candidate as a human tumour virus.

Turning finally to the oncornaviruses which have so intensely been discussed at this conference, it seems evident that most animal species contain either endogenous or exogenous oncornaviruses. The evidence for the presence of similar viruses in human beings especially in human leukaemia or in human breast cancer, is, however, scanty. The risk of laboratory contaminations is also great. There are, however, a few isolates and some will hopefully be discussed at this meeting. Recently viral sequences closely related to sequences of the genome of baboon virus have also been demonstrated in cellular DNA in several leukaemic patients. In addition, sequences of murine leukaemia virus have recently been found in DNA of a few patients.

It has been suggested, however, that synthesis of complete infectious and endogenous RNA tumour viruses, if indeed such viruses exist, must be a rare or non-existent event in humans. Considering the existence of such viruses, however, the recent finding that, for instance, each individual case of murine leukaemia probably is caused by a slightly different virus, complicates the matter. The main information concerning the tumour formation may actually lay in the cells and the oncornaviruses may only pick up these sequences. They may have quite different functions in the cell and the organism and the oncogenic property might be just a coincidence. Also the question of the mode of transmission of these viruses might have to be re-evaluated considering for instance the presence of oncornaviruses in insects.

Another finding of interest, although probably not unexpected, is that there does not seem to be any homology between the *src* gene of the C-virus particles and the genome of for instance SV40 virus: — are there many ways to make a key — are there many keys to the same lock or are there many keys and many locks?

The acute leukaemia viruses of chicken might bring some new interesting aspects on the genome of the oncornaviruses and the mechanism of transformation.

When considering all the data, presented at this conference, one has the impression that very much has been learnt about the viruses mentioned here, about their structure, properties and animal models but that very little is known about the relation of these viruses to human tumours and one has therefore to hope for new approaches and new methods in order to solve the question of the role of viruses in human cancer.

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THE MOLECULAR BIOLOGY OF ADENO-, POLYOMA- AND SV40-VIRUSES: RECENT DEVELOPMENTS*

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In my own judgement, the progress which has been made in the field within the recent one-and-a-half years, ever since we have met on the occasion of the last European Tumour Virus Meeting is quite considerable. There are contributions which have an impact not only on tumour virology but also on molecular biology in general.

I would like to mention first the discovery made with adenovirus RNA that a mature messenger RNA is composed of regions which are non-contiguous on the genome. Such an RNA molecule is most likely brought about by removal of the intervening regions from a larger RNA molecule and subsequent splicing together the so-called leader-sequences and the body of the messenger RNA. Via this mechanism there appear in mammalian cells different messenger RNA species sharing common leader sequences. The same discovery has been made in the case of SV40 and polyoma viruses. Methods have been developed to measure the size of both the leaders and the bodies of various mature poly A containing cytoplasmic messenger RNAs. For example, using the R-loop technique, displacement loops of single-stranded DNA are formed between double-stranded DNA and mature messengers which can be visualized in the electron microscope and which permit the localization of the leaders on the DNA. Another technique involves the use of S_1 nuclease to hydrolyse the single-stranded displacement loop between single-stranded DNA and mature mRNA in hybrids. By comparison of the electrophoretic mobility of the native and denatured structures both before and after S_1 -treatment one can define the length of the leaders and of the bodies of various mRNAs.

In case of SV40 there exist two late mRNA species of 19 and 16S, with common leader sequences. It was also shown that there are two early mRNA species sharing a common leader region.

Localization of the deleted intervening sequences between leader and body of one of the early messengers which map between position 0.54 and

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0.59 fits nicely with the observation that this region of the genome is dispensable for the vegetative growth of SV40. The use of viable deletion mutants has permitted the allocation of biological functions on the physical map of the genome.

The mutants bearing deletions between position 0.54 to 0.59, although being viable, affect transformation in that the cells appear to be more anchorage dependent than wild type transformants. Furthermore, their actin network is better developed than in wild-type transformed cells. Most interestingly, there is a direct correlation between the extent of deletion and the size of the so-called little t-antigen. It may be recalled that there is now substantial evidence for the existence of two SV40-tumour antigens, 17 and 90 kilodaltons in size and termed little and large T-antigen. In parentheses I might mention that in the case of polyoma there appears to exist in addition, a medium-sized T-antigen.

To link together the various recent observations it is clear from the investigation of the spliced early messengers, from the knowledge of the molecular weights of the little and large T-antigens and from the study of the biological functions which are affected by defined deletions, to which regions of the physical map both the coding sequences for little and large T-antigen are confined. While the large T shares common sequences with the little t, it consists, in addition of the polypeptides coded for, from map position 0.53 to 0.17. While large T is involved in the initiation of DNA replication, little t seems to be responsible according to the few data available, for some aspects of transformation.

The correlation between defined regions of the viral genome, their transforming ability and the polypeptides that are encoded by them appears to be even more advanced in the case of adenoviruses, in which case transformation has been accomplished with restriction enzyme cleavage fragments of various sizes. The 7.5% terminal of the left end of adeno-5-DNA suffices to transform cells. Even the 4.5% left end terminal is capable of conveying to the cells at least some parameters of transformation. It has been shown by the *in vitro* translation of messenger RNAs derived from cells transformed by the 7.5% end that at least 6 polypeptides are located within this region of the genome.

What has to be done obviously is to fill our gap of knowledge concerning the biological functions of the proteins that are relevant to transformation in the case of both the adenoviruses and the SV40-polyoma-subgroup viruses. Since the complete sequence of the SV40 DNA is now available, it will be possible at that point to ascribe a biological function of eminent importance to a defined DNA sequence.

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RECENT PROGRESS IN THE STUDY OF AVIAN ONCORNAVIRUSES*

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The high number of publications reporting interesting new data on avian oncornaviruses and their interaction with infected cells which have appeared in 1977 and 1978 precludes any detailed review. One may only attempt to summarize those which seem to be the most interesting in the biased light of one's major interests. The data which have thus been chosen relate to the structure of the viral genome and viral genetics, to the mechanisms of viral replication, or absence of replication, in permissive and nonpermissive cells, and to the mechanism(s) of cell transformation, the crucial problem from the point of view of cancer.

1. Structure of the viral genome and viral genetics

It is now well established that the viral genome consists of a single stranded RNA molecule of about 3×10^6 daltons with a total coding capacity of $2.5\text{--}3 \times 10^5$ daltons of polypeptide [1]. In the virion, two identical molecules are hydrogen bonded near their 5' ends to form 70S RNA. This region has been sequenced and a detailed model of the dimer linkage has been proposed [2].

It has been established [3–6] that there exists a terminal redundancy of nucleotide sequences at the 5'-capped and 3'-polyadenylated extremities of the viral RNA comprising 6–20 bases depending on the viruses. Moreover, recombinants of viruses with different terminal sequences have both terminal sequences derived from one parent [5, 6]. This suggests that the function of the redundant terminal sequences is to allow the polymerase (reverse transcriptase) which synthesizes viral cDNA to jump from the 5'-end to the 3'-end of the same circularized RNA molecule or its associate in the transcription process. The genetic linkage of the terminal sequences also supports the view that recombination occurs between circular proviral DNAs [5].

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There is now general consensus about the genes present on the viral RNA and their order, from the 5'-end to the 3'-end; i.e., *gag*, *pol*, *env* — coding respectively for the *gs* p proteins, the polymerase, and the envelope glycoproteins — in nondefective ALV or transformation defective (*td*) ASV, and the same genes plus *src* — the transformation gene — in ASV. The respective sizes of these genes are over 3000 nucleotides for *gag* and for *pol*, about 2300 nucleotides for *env* and 1500–1900 nucleotides for *src*. In addition, there is a common (*c*) sequence of 450–750 nucleotides between the 3'-polyadenylated extremity and *env*, for ALV, or *src*, for ASV [7]. Therefore, if *src* is a host cell gene recombined with an ALV genome (cf. further) it is not simply added terminally to this genome but inserted inside it.

Deletions of various sizes in the replication genes (*gag*, *pol*, *env*) appear to account for the helper requirement of defective ASV or ALV such as AEV, AMV, MC29 and MH2 [1]. In the case of MC29, the virion RNA has been shown to have only about 5700 nucleotides against 9–11 000 for nondefective ALV or ASV, of which only 70% are homologous to those of the latter viruses and 30% distinct. This distinct sequence, or part of it, might be a transforming gene equivalent to *src* in ASV [8]. Defectiveness of viral replicative genes has also been demonstrated in certain endogenous viruses which require a helper for activation [9], and also in ASV in some nonpermissive, noninducible transformed mammalian cells [10, 11].

2. Mechanisms of viral replication

Except the mechanism of integration of proviral DNA into cellular DNA, the early steps of viral replication are relatively well known. The recent data of interest concern later steps, i.e., transcription and translation of the integrated proviral DNA.

Transcription of proviral DNA. It has been shown [7, 12] that cDNA of nondefective ASV hybridizes with 3 classes of poly(A)-containing RNA in ASV-infected cells, of sizes 39S, 28S and 21S. The genetic information carried by these RNAs has been determined by hybridization with cDNA sequences specific of the viral genes [7, 12] and their function by micro-injection and/or *in vitro* translation [cf. 13]. In short, 39S RNA carries all the viral sequences and either goes into virions or serves as mRNA for the *gag* and *pol* genes, whereas 28S RNA carries *env* and *src* sequences, plus 3'-end *c* sequences, and serves as mRNA for *env* while 21S RNA carries only *src* and *c* sequences and is the *src* mRNA. In the case of nondefective ALV or *td* RSV deletion mutants, one finds 35S genomic RNA which serves as mRNA for *gag* and *pol*, and 21S *env* mRNA; *env*-deletion mutants of ASV also have a smaller 35S genomic RNA serving as mRNA for *gag* and *pol*, plus 21S *src* mRNA, but no 28S *env* mRNA.

In uninfected chicken cells which shed RAV-O the situation is presumably similar to that in cells infected with nondefective ALV, whereas in nonproducer cells carrying a defective endogenous virus with sequences missing both in *gag* and *pol* [9], two major RNA species have been found: 31S genomic RNA and 21S *env* mRNA. In *gs*⁺, *chf*⁺ cells, expressing *gag* and *env* antigens in appreciable amount, the amount of the viral RNAs is 100 times less than in exogenously infected cells, but 100 times higher than in uninfected *gs*⁻, *chf*⁻ cells expressing the viral antigens at a much lower (for *gag*) or undetectable (for *env*) level. This difference in the level of transcription, in the case of both nondefective and defective endogenous viruses, might be related to the presence of an adjacent cellular gene, i.e., to the site of integration of proviral DNA in the cellular DNA. In favour of this model, it has been found that transfection of chicken cells with DNA from chicken cells producing RAV-O at a very low level succeeds only when the DNA is sheared to the size of the virus genome [14].

ASV-transformed nonpermissive (mammalian) cells also contain viral-specific RNA in variable amount comparable to that in uninfected chicken cells [cf. 13]. This suggests that the same type of transcriptional control might exist in both cell types. Indeed, transfection of chicken cells with the DNA from ASV-transformed hamster BHK cells with a low level of inducibility and only a few viral RNA copies succeeds only with genome-sized DNA, whereas transfection with the DNA from highly inducible cells with 10 times more viral RNA copies succeeds also with larger DNA [15]. In other ASV-transformed BHK cells and their phenotypic revertants, viral 35S RNA and 21S *src* RNA have been found in the nucleus and the cytoplasm and on polyribosomes, but *src* RNA has been shown to be 10 times less abundant in revertant than in transformed cells [16]; however, no such difference has been observed in other ASV-transformed cells expressing viral RNAs in the same way and to the same degree [17]. Another study has shown that, in addition to 35S and 21S viral RNAs, the nucleus of ASV-transformed hamster cells can contain 47S viral-specific RNA that might include cellular sequences [18].

In all cases listed, the generation of viral mRNAs smaller than genomic RNA may be explained in two different ways: (i) processing of the genome-sized RNA to smaller species subsequently capped at their 5'-end [13], or (ii) "splicing" together of segments of RNA as in the case of adenovirus mRNAs [19]. In favour of the second model is the finding that all viral mRNAs contain identical or similar "leader" sequences at their 5'-end [12, 20].

Translation of viral RNAs. In permissive cells infected with nondefective ASV, the genome-sized RNA is translated to give either Pr76, the 76 000 dalton precursor of the viral *gs* (*gag*) proteins [13], or Pr180, the larger 180 000 dalton precursor of the polymerase which also contains the peptides of Pr76 but does not seem to be its precursor [21]. As Pr76, Pr180 is also made *in vitro*

[22]. It is known [13] that cleavage of Pr76 gives rise to *gs* proteins p19, p27, p12 and p15 (in that order from the 5'-end of *gag* RNA) and might occur at the time of virion budding at the cell membrane, whereas Pr180 might be cleaved to give the polymerase only inside virions [21]. Cleavage of Pr76 may be due in part to p15 since it can cleave Pr76 made *in vitro* which normally is not cleaved as *in vivo* [23]. On the other hand, the fact that genome-sized RNA may direct the synthesis of both Pr76 and, less often, Pr180 might be due to leakiness of a stop signal between *gag* and *pol* or to the loss of this signal in some RNA molecules.

From this point of view, cells transformed by the replication defective ALV MC29 which lacks *gag* sequences for p15 and presumably part of *pol* contains no Pr76 but only a 110 000 dalton *gag* polyprotein which turns over rapidly but is not processed to *gs* structural proteins [24]. Similarly, in uninfected chicken cells requiring helper virus for activation of the endogenous virus, the *gs* antigenic determinants have been found on a 120 000 dalton polyprotein containing RAV-O specific peptides p19, p27 and p12, plus several peptides of reverse transcriptase, but no p15 peptides [25]. In contrast, cells producing RAV-O synthesize and process Pr76.

ASV-transformed nonpermissive mammalian cells also contain Pr76 presumably programmed by genome-sized RNA, but in much lower amount than permissive cells, which may be due to the lower level of transcription of viral RNA. In addition, there appears to be a block of cleavage of the precursor polyprotein which is released only following fusion with permissive cells [cf. 13]. Hence, the latter cells may contain a factor necessary for the cleavage which lacks in the nonpermissive cells. Yet, some cleavage of Pr76 must occur in semi-permissive mammalian cells.

As concerns translation of the 28S or 21S *env* RNA of ASV or ALV, it gives rise to a 90 000 dalton precursor (gpPr90) of gp85 and gp37 which is subsequently processed to these envelope proteins [26, 27]. This precursor can also be made *in vitro* [28] but does not appear to have been found in uninfected chicken cells or transformed mammalian cells.

Knowledge of the translation product(s) of the 21S *src* RNA is less advanced; yet, interesting data have been obtained recently. First, a transformation-specific antigenic protein of 60 000 daltons has been found by one team in ASV-transformed cells [29] and also obtained *in vitro* in the presence of *src* RNA [30]. A second team [31] has obtained, instead, *in vitro*, two polypeptides of 25 000 and 17 000 daltons, respectively, while a third one [32] has obtained both these polypeptides and a 60 000 dalton polypeptide which might all originate from different readings of *src* RNA. Moreover, this same team has found that the 25 000 dalton polypeptide comprises five components with different isoelectric points and the 17 000 dalton polypeptide two ones. Yet, it is not known whether all these are also made *in vivo*.

3. Cell transformation

In the case of ASV, it is now well established by the study of transformation defective (*td*) deletion mutants and temperature-sensitive (*ts*) mutants defective only for transformation, that virus-induced cell transformation depends entirely on the viral *src* gene which is also required for the maintenance of the transformed phenotype [33, 34]. This phenotype consists of a number of interrelated parameters amongst which morphological transformation, alteration of growth behaviour and modifications of the cell surface and of intracellular transport of metabolites (notably hexoses) appear to be the most important [cf. 34]. All the important parameters of cell transformation have been shown to be governed by *src* inasmuch as they are reversible in cells transformed by *ts* mutants after shifting to the restrictive temperature. Yet, depending on the parameters, the protein(s) coded for by *src* could either interact directly with targets located at the cell periphery, notably at the cell surface, or act centrally to alter the expression of viral genes. From this viewpoint it has been shown that morphological transformation of cells infected with a *ts* mutant can occur even after enucleation and must, therefore, result from the direct action of a *src* protein on a peripheral cellular target [35]. Experiments with another *ts* mutant have yielded results suggesting that the *src* product(s) acts directly on the surface modulating assembly composed of microtubules, microfilaments and surface receptors which has been postulated to regulate surface receptor mobility and cell growth [36]. Yet, other studies have shown that transformation of chicken cells by ASV is accompanied by important alterations of the levels of translatable mRNAs for cell membrane proteins and collagen precursors, i.e., changes in the activity of specific cellular genes [37]. The same team has proposed that the alteration associated with morphological transformation (rounded cell shape, loss of surface proteins and carbohydrates, increased agglutinability by lectins, dissociation of microfilament bundles) are separable from alterations of growth properties (loss of anchorage dependence and density-dependent inhibition of growth, and tumorigenicity) [38]. Along this same line, it has been found that certain ASV *ts* mutants are only partially defective for transformation at the restrictive temperature and retain the wild type phenotype for the parameters related to cell growth (loss of anchorage dependence and density-dependent inhibition of growth) [39]. Similarly, it has been shown in another system, chick embryo neuroretinal cells, that the multiplication of resting cells is stimulated by ASV *ts* mutants at restrictive temperature in the absence of morphological transformation and increase of hexose uptake [40]. Furthermore, it has been possible to isolate ASV *td* mutants able to stimulate cell multiplication in the absence of transformation at normal temperature [41]. Hence, all recent data suggest that the ASV *src* gene controls two sets of interrelated transformation parameters

comprising respectively the parameters related with morphological transformation and the parameters related to cell growth. Control of these parameters might be achieved in two ways. On the one hand, it could depend on a unique protein which may be the 60 000 dalton protein found *in vivo*, the size of which fits well with the coding capacity of *src*. In that case this protein should be multifunctional and able to interact with at least two distinct sites within the cell. Moreover, in the case of the growth parameters, the *src* protein could interact with the expression of cellular genes. Alternatively, the two sets of parameters could be controlled by two different *src* products which could be the 25 000 and 17 000 dalton proteins made *in vitro* and of which the first may derive from the 60 000 dalton protein. [32]. All these three proteins, plus the other ones made *in vitro* might also control individual parameters of the transformed phenotype [31].

Another interesting advance is the finding of *td* mutants of ASV with incomplete deletion of *src* [42, 43]. Some of these mutants have been shown to recombine with a *ts* mutant, and some also to give rise to sarcomas *in vivo* from which apparently wild type ASV has been recovered [44]. This suggests that a *td* mutant might recover its transforming capacity by recombination with cellular DNA.

This raises the problem posed by the important finding in normal chicken cells, and also cells of other neighbour and even distant species, of a *src* sequence homologous to viral *src* [45]. This suggests that ASV may have arisen by recombination of ALV with the endogenous *src*, eventually followed by some mutation of this gene. Alternatively, insertion of *src* by the provirus in an aberrant location in the cell genome might suffice to elicit unregulated transcription and translation of its information resulting in neoplastic transformation. From this point of view, the finding in MC29 RNA of sequences unrelated to RNA sequences of other ALV or ASV and amounting to about the size of *src*, although entirely different from *src* sequences, raises the possibility of recombination of ALV with other cellular sequences which, as *src*, might be involved in the regulation of cellular growth. Indeed, it has recently been observed with another avian acute leukaemia virus, AEV, that the viral RNA, of about the same size as that of MC29, comprises a set of nucleotides distinct from both ALV and *src* sequences but present as *src* in the genome of normal chicken cells, and also cells of neighbour species [46]. Hence, ALV giving rise to acute leukaemia as well as ASV (some of these ALV are also able to give rise to other types of tumours) may have acquired their transforming capacity by incorporation of cellular information involved in the regulation of cell growth. If so, the study of the functions of cellular genetic material incorporated into the viral genome may give an insight into the mechanism of regulation of growth of normal cells and, at the same time, allow to understand the mechanism(s) of neoplastic transformation.

Returning to transformation by ASV, there remains the problem of the role in this transformation of the virus-specific surface antigen, or tumour-specific surface antigen (TSSA) found on all cells transformed by these viruses and which may be, at least in part, a product of the *src* gene. This antigen is accompanied by another antigen which cross reacts, in the case of quail cells, with an antigen also present on methylcholantrene-transformed cells [47]. Also, treatment with 5-bromodeoxyuridine (BrdU) of chick embryo or quail embryo cells elicits the appearance of a cell surface antigen detected by anti-serum to TSSA-carrying rat cells (XC cells) [48]. This antigen might be the same as that found on methylcholantrene-transformed cells or another antigen derepressed by BrdU. In any case, these findings suggest that the *src* gene may direct the synthesis of a new surface antigen and elicit the appearance of cell-coded antigens which might play a role in the alteration of the regulation of cell growth. On the other hand, it has been shown that TSSA, or at least a TSSA-related antigen of about 40 000 daltons is released in the medium by ASV-transformed cells [49]. ASV-transformed cells also release into the medium another protein of higher molecular weight which may play a role in the maintenance of transformation since it enhances transformation of chicken cells by ASV partially defective in its transformation capacity or residual transformation by an ASV leaky *ts* mutant [50]. This protein, which may not be unique, might be coded for by the cell since it is also shed by cells transformed by other viruses of transforming agents, or by untransformed cells established *in vitro*. The activity of this, or these, proteins has also been demonstrated recently in the plasma of patients with leukaemia or solid tumours, but not of patients with other diseases or healthy controls [51]. Hence, the expression of *src* can elicit alterations also present in cells transformed by other oncogenic viruses or agents, or in spontaneously transformed cells.

4. Other problems

In relation with the problem of recombination of the ALV genome with cellular *src* or other genes involved in the control of cellular morphology and growth, there is also the problem of recombination of the viral genome with other cellular genes and transduction of these genes in infected cells of the same or other species. Earlier investigations [52] have shown that repeated passages of ASV in duck cells may result in the picking up by the viral genome of duck nucleotide sequences. Similarly, it has been observed recently [42] that *td* mutants of certain ASV strains carry in their genome, in the place of *src* sequences, cellular sequences of 600–700 nucleotides corresponding to a coding capacity of about 20 000 daltons of polypeptide. Hence, ALV might become transducers of cellular genetic information distinct from *src*. Checking this possibility requires the finding of some genetic marker(s) controlled by the cellular sequences.

From the general aspect of genetic studies of avian oncornaviruses and their action on infected permissive or nonpermissive cells, one should finally emphasize three methodological advances which might prove of major importance for future investigation. These are (i) the microinjection of viral mRNAs into cells [53]; (ii) the complementation rescue of virus from transformed non-producer cells by polyethylene-glycol-mediated cell fusion [54]; and (iii) marker rescue with fragments of DNA from virus-infected cells [55]. All these methods, together with *in vitro* translation of viral mRNAs should become routine instruments of future integrated genetical and biochemical studies in the field.

N. B. Since this review had been completed, there appeared another paper [56] reporting that the 60 000 dalton protein postulated to be the *src* gene product possesses a protein kinase activity and might, therefore, exert its action by phosphorylating some specific target protein(s).

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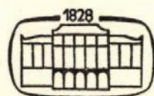
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HAEMAGGLUTINATION-INHIBITING ANTIBODIES TO B.K. VIRUS IN HUNGARY*

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(Received December 8, 1977)

Antibody inhibiting the haemagglutination by B.K. virus was found in 64% of the serum samples collected from 949 subjects in Hungary. The frequency of seropositivity was the lowest (17%) among infants 1 and 2 years of age and the highest (93%) in the age group 16 to 20 years. The subsequent age groups showed a slow continuous decline. The age-distribution curve drawn on the basis of the geometric means of titres has two peaks, one between 3 and 5 years of age and another between 21 and 30 years of age, reaching 6.74 and 7.05, respectively, as expressed in terms of $\log_2$ units. The prevalence of haemagglutination-inhibiting antibody to B.K. virus in Hungary and its distribution by age are similar to those reported from other European countries.

B.K. virus [1], a recently described papovavirus, has proved oncogenic in neonatal hamsters [2, 3] and capable of causing malignant transformation of cells *in vitro* [4–8]. Consequently, its seroepidemiological properties deserve attention. Antibodies to B.K. virus were found to be common in the populations of several countries including England [9], Finland [10], Italy [11, 12], USA [13] and other countries [14, 15]. In this work, the presence of B.K. virus antibodies in Hungary has been studied and analysed by age.

Materials and methods

Serum samples. Serum samples were collected between January, 1976, and July, 1977, from 949 subjects (458 males and 491 females), including carefully selected surgical patients and healthy subjects. The age distribution of the donors is shown in Table I. The sera were stored at -20°C until tested.

Virus. Samples of the prototype strain of B.K. virus were kindly supplied by Dr. M. PORTOLANI (University of Bologna, Bologna, Italy) and Dr. S. D. GARDNER (Central Public Health Laboratory, London, England). The virus was propagated in Vero cell monolayers. MEM enriched with 10% calf serum was used as growth medium. The cells were separated from the glass wall by treatment with 0.25% trypsin solution and the infected cultures were maintained in MEM containing 1% calf serum. The multiplicity of infection was 0.01 fluorescent-antibody-focus-forming unit/cell [16]. The medium was changed weekly and the virus was harvested in the third or fourth week postinfection.

Antigen. Haemagglutinin was prepared as described by MÄNTYJÄRVI *et al.* [17, 18]. Infected cells detached from the glass wall were frozen and thawed in the maintenance medium to which 0.1% Nonidet P-40 (Shell Chemical Co., USA) had been added. The lysate was sonicated for 60 s three times in Branson Sonifier 303 (Branson Europe N.V., Soest, The Netherlands) and the residual debris were removed by centrifugation at 2000 *g* for 15 min.

* Parts of this paper were read at a Conference of the Hungarian Society of Microbiology, Gyula, August, 1977.

The supernatant was extracted twice with fluorocarbon (Genetron 113, Allied Chemicals, New York, USA). The partially purified haemagglutinin thus obtained was titrated and its 2 ml samples were stored at -20°C until used.

Serological tests. Micromethods [19] were performed in wells of the Microtiter tray (Labor MIM, Budapest, Hungary). The optimum circumstances for B.K. virus haemagglutination (HA) are described in another paper [20]. Nonspecific HA inhibitors were removed from serum samples by pretreatment in 0.01 M NaIO_4 dissolved in distilled water [18]. For haemagglutination-inhibition (HI) titration 25 μl serum and 50 μl NaIO_4 were measured into each well, the reagents were mixed by gentle shaking and the trays allowed to stand at room temperature for 30 min. Subsequently, 175 μl glycerin were added to each mixture. The tenfold serum dilution thus obtained was used as working-dilution for preparing a halving dilution series. HSAG buffer [19] pH 6.0 was used as diluent, 8 units of haemagglutinin were added to each well and the system was allowed to react at room temperature for 30 min. Then human group O erythrocytes were added in 0.5% suspension and the trays were kept in a wet chamber of 4°C overnight before the results were read. Simultaneous controls were performed with each titration.

Table I

Age incidence of HI antibodies to B.K. virus

Age group	No. of sera tested	Positive sera, per cent
0— 6 months	104	59
7—11	77	38
1— 2 years	140	17
3— 5	64	38
6—10	66	78
11—15	66	80
16—20	66	93
21—30	99	91
31—40	67	82
41—50	71	81
51—60	69	75
>60	60	71
Totals	949	64

Results

Prevalence of HI antibodies to B.K. virus in different age groups

Of the 949 serum samples 604 (64%) proved to contain HI antibodies. The samples were obtained from 12 age groups each consisting of 60 to 140 subjects. The frequency of positive sera in infants under 6 months (59%) reflects this percentage. The lowest percentage was found in children 1 and 2

years of age (17%). This was followed by a sharp rise up to 93% found between 16 and 20 years of age. Although the frequency of antibody prevalence was declining over this age, it did not fall below 70% even beyond 60 years.

Antibody level in different age groups

Geometric means. The geometric means for the titres in different age groups, expressed in $\log_2$ units, are shown in Fig. 1 and Table II. There was a slight decline in mean values during the first year of life and a sharp rise after 3 years of age. After another slight decrease, the highest value was reached in the age group from 21 to 30 years, and this was followed by a considerable fall parallel with age.

Frequency of high-titre sera. The frequencies for sera with titres $\geq 1:320$ are also shown in Table II. In the first three years of life, the frequency of high-titre sera remained below 3%. In the 3 to 5 years age group, however, three, two and two serum samples had titres of 1:320, 1:640 and 1:1280, respectively. A peak was reached between 6 and 10 years of age (21%) and a subsequent decline was followed by a second, still higher, peak in the age group from 21 to 30 years of age (36%). In the latter age group 16, 16 and 4 sera had titres of 1:320, 1:640 and 1:1280, respectively. After a gradual decline, no high-titre sera were found beyond 60 years of age; even sera with titres 1:160 and 1:80 were as few as 1 and 4, respectively. For comparison, 20 and 8 sera had such titres in the age group from 21 to 30 years.

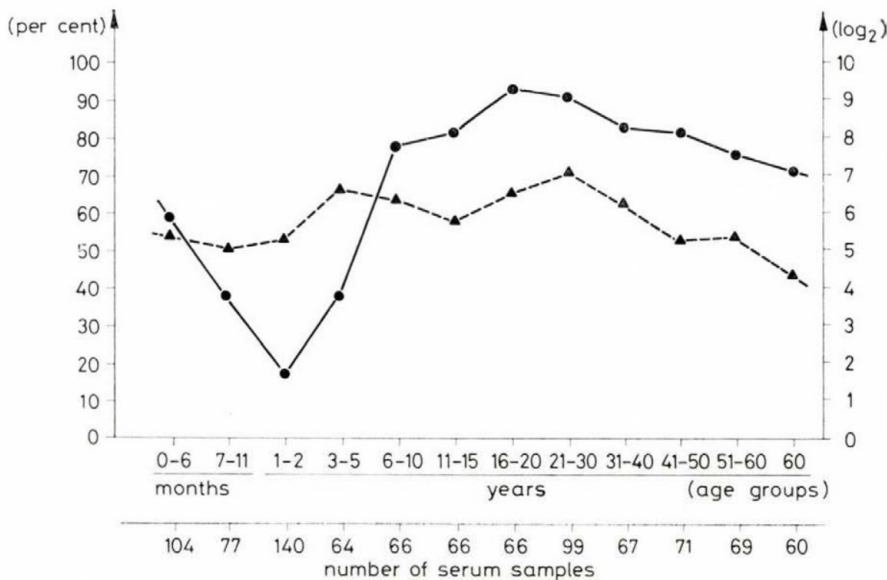


Fig. 1. HI antibodies to B.K. virus. Age incidence and geometric mean of titres: ●—● positive sera, per cent; ▲—▲ geometric mean in $\log_2$ units

Table II
*High-titre sera and geometric means for HI titres
 in different age groups*

Age group	No. of sera	Sera with titres $\geq 1:320$		Geometric mean $\log_2$
		No.	per cent	
0— 6 months	104	2	1.9	5.40
7—11	77	1	1.3	5.08
1— 2 years	140	4	2.8	5.24
3— 5	64	7	10.9	6.74
6—10	66	14	21.2	6.36
11—15	66	7	10.6	5.87
16—20	66	16	24.2	6.48
21—30	99	36	36.3	7.05
31—40	67	16	23.8	6.24
41—50	71	6	11.2	5.34
51—60	69	2	2.8	5.46
>60	60	0	0.0	4.40
Totals	949	111	11.7	5.90

Discussion

We have succeeded in demonstrating the occurrence of B.K. virus infections in Hungary. The present results provide further evidence for the wide occurrence of the virus.

A comparison between results obtained in different laboratories is, however, limited owing to (i) methodical diversities, namely, nonspecific inhibitors were removed by different methods [9–11, 13, 15], and (ii) inconsistent age-groupings. Comparable figures are those indicating the percentage of antibody-containing sera calculated for the total number of sera tested in the respective study, though the samples examined were only approximately age-matched. The 64% incidence found by us agrees well with the 62% ($n = 409$) found in England [9], the 58% ($n = 203$) found in Finland [10], and the 67% ($n = 453$) and 65% ($n = 582$) found in Italy [11, 12]. In the USA, 74% of the tested 334 serum samples proved to be seropositive [13]. In Japan, TAGUCHI *et al.* [15] found in this respect no difference between pregnant and nonpregnant women. BROWN *et al.* [14] collected 1544 serum samples from many parts of the world.

They found antibodies reacting with the B.K. virus everywhere except in closed populations, e.g. red Indians in Brazilian jungles, where such antibodies were found in 2 to 6% if at all.

In spite of the different age-groupings, it may be stated that the tendencies demonstrated by us for Hungary are similar to those reported for other countries. In the samples of PANÀ *et al.* [12], similarly to ours, the 1 and 2-years old children showed the lowest incidence of positive sera (17%). SHAH *et al.* [13] reported a higher prevalence (33%), but these authors examined as few as 15 samples in this age group. Around 10 years of age, the percentages are much higher: in Hungary 78%, in England 83% [9], in Finland approximately 63% [10], in Italy 76% and 73% [11, 12]. SHAH *et al.* [13] reported 100% for those aged 10 to 11 years. The curve in Fig. 1 shows a single peak at 16 to 20 years (93%) and the figure for the age group from 21 to 30 years is only slightly lower (91%). The Finnish workers as well as one of the Italian teams [12] found the highest prevalence (83%) between 21 and 30 years while according to the other Italian team [11] the peak (84%) was reached between 16 and 25 years. Over 50 or 60 years, mostly 60 to 65% of the samples proved to be positive.

We have chosen periodate treatment because most of the European workers, e.g. [11, 12] and, in their late investigations, also MÄNTYJÄRVI *et al.* [10] applied the same method. GARDNER *et al.* [1] used RDE, and the figures reported by them are comparatively high. SHAH *et al.* [13], who, like BROWN *et al.* [14], absorbed sera with acetone, pointed to some disadvantages of RDE pretreatment, namely residual nonspecific inhibitors were demonstrated in some of the RDE-pretreated sera. The results published by SHAH *et al.* [13] are in good accordance with those obtained by others in KIO_4 -pretreated sera. TAGUCHI *et al.* [15] found no differences in differently pretreated (RDE periodate, acetone, kaolin) and untreated sera. Therefore, they applied no pretreatment in routine work.

PANÀ *et al.* [12] regarded serum titres over 1 : 160 as an indicator of fresh infection. We prefer the use of the geometric means of titres as an index in comparative age-distribution studies. Since use of this index is uncommon in literature, we have chosen the percentage frequency of high-titre ($\geq 1 : 320$) sera as a basis of comparison. Figure 1 shows a peak (21%) at 6 to 10 years and another (36%) at 21 to 30 years. The geometric mean as index shows two peaks, but the first occurs at 3 to 5 years instead of 6 to 10 years. The age distribution published by PANÀ *et al.* [12] has peaks at 3 to 4 years and at 21 to 30 years. Similar tendencies are shown by the figures of PORTOLANI *et al.* [11] (peaks at 3 to 5 and 16 to 25 years). In the samples examined by MÄNTYJÄRVI *et al.* [10] the first age group cannot be taken into account in this respect, but the peak at 16 to 20 years deserves consideration. The samples examined by GARDNER [9] and SHAH *et al.* [13] show no similar peaks.

The two peaks of the curves representing the age distribution of high titres is suggestive of the existence of two ages of life at which exposure to B.K. virus is comparatively high. The first peak might correspond to transmission with urine whereas the second peak might be attributed to sexual contact. These questions need further investigations and more detailed age-grouping.

It may be concluded that HI antibodies to B.K. virus are widely present in the population of Hungary. The high frequency of high antibody titres in two age groups suggests that fresh infections are commonest between 3 and 5 years and between 21 and 30 years of age. A correct comparison of results published from different laboratories needs identical pretreatment of sera and identical age-grouping.

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B.K. VIRUS HAEMAGGLUTININ*

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Among the widely applied buffered media, the HSAG (hepes-salt-albumin-gelatin) medium at pH 5.75–6.25 was found to be the most favourable for B.K. virus haemagglutinin titration. The optimum temperature was at 4 °C. The haemagglutinin was not affected by temperatures up to 37 °C, pHs between 5.5 and 9.5, and NaCl concentrations between 0.063 M and 2.56 M. When incubated at 56 °C, the haemagglutinin shows a time and pH dependent decline in titre. No significant time dependent titre fall occurred at 56 °C if NaCl molarity was varied between 1.31 and 2.56.

The first isolation of B.K. virus, a human Papovavirus was reported by GARDNER *et al.* [1] in 1971. The strain had been isolated from the urine of a recipient of renal transplant. Since then several identical strains have been isolated from humans [2–6]. The basic properties of the haemagglutination reaction caused by the virus were described by GARDNER *et al.* [1], MÄNTYJÄRVI *et al.* [7] and TAKEMOTO and MULLARKEY [8]. Accordingly, the haemagglutinin is virion-associated and its highest titres can be reached with human "O" erythrocytes at 0 °C in the pH range between 6.1 and 7.4.

The aim of the present work was to find the composition and pH of the reaction medium and the incubation temperature most favourable for the titration of B.K. virus haemagglutinin, and to determine its resistance to chemical and physical factors.

Materials and methods

Virus. Gardner's prototype strain was propagated in Vero cell monolayers [9].

Haemagglutinin. Infected Vero cultures were incubated at 37 °C. The cells were harvested together with the medium and treated with the non-ionic detergent Nonidet P-40 (Shell Chemical Co., USA) as described by MÄNTYJÄRVI *et al.* [7]. The mixture was sonicated (Branson Sonifier 303; Branson Europa N.V., Soest, The Netherlands) for 60 s three times, then centrifuged at 4 °C at 2000 g for 15 min and extracted with fluorocarbon (Genetron 113, Allied Chemical, New York, USA) twice. The partially purified haemagglutinin thus obtained was stored at –20 °C until used.

Erythrocytes. Human group-O erythrocytes were kept in Alsever solution and washed three times immediately before use.

Buffers and other solutions. Dextrose–gelatin (DG) solution for washing erythrocytes: 1% glucose, 0.06% gelatin, 0.4 mM CaCl₂, 0.5 mM MgSO₄ in 0.14 M NaCl solution. Phosphate

* Parts of this paper were read at the Meeting of the Hungarian Society of Microbiology, Gyula, August, 1977.

and veronal buffers (0.025 M) were prepared in 0.15 M NaCl in the pH range from 5.5 to 8.5. Buffered DG solutions: DG-glycine-HCl (DGGI) solution pH 1.67-3.65; DG-Na-citrate-HCl (DGC) solution pH 4.60-7.50; DG-glycine-NaOH (DGGII) solution pH 8.55-10.50; DG- $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ (DGP) solution pH 5.48-8.40; DG-veronal-HCl (DGV) solution pH 5.50-8.40; DG-Tris-HCl (DGT) solution pH 6.50-9.40. In all these solutions 0.025 M or 0.05 M buffer system was incorporated. In addition, HSAG (hepes-salt-albumin-gelatin) buffers of pH 5.75, 6.0 and 6.25 were used [10].

Haemagglutination (HA) reaction. Microtitrations were performed essentially as described by MÄNTYJÄRVI *et al.* [7]; 0.5% suspension of human group-O erythrocytes and, as medium, 0.025 M buffer systems or HSAG were used. The temperature of incubation was varied. The reaction was read immediately when the control erythrocytes had settled.

Measurement of the resistance of B.K. haemagglutinin to pH, salt and temperature. Haemagglutinin containing 20 480 HA units/ml was exposed to 0.063 to 2.653 M NaCl concentrations at pH 6.0 and at temperatures of 4, 20, 37 and 56 °C. Three ml samples of haemagglutinin were mixed with an equal volume of distilled water or NaCl solution at molarities of 0.15, 1.25, 2.5 and 5.0 M. If it was necessary, the pH was adjusted to 6.0. Four series of these mixtures were incubated at the temperatures indicated above. A sample was taken from each mixture immediately before incubation and after an incubation for 1, 6, 24 and 168 hr. The samples were titrated in HSAG medium at pH 6.0.

To measure resistance to pH, 5 ml samples of haemagglutinin (20 480 HA units/ml) were mixed with an equal volume of one of the following 0.05 M buffer solutions: DGP pH 5.45, DGT pH 6.5, DGT pH 7.55, DGT pH 8.45 and DGT pH 9.4. Four series of these mixtures were incubated at the temperatures indicated above. Samples were taken immediately before and after an incubation for 1, 4, 26 and 72 hr. The pH was adjusted to 6.0 before titration.

Results

HA titres under different conditions

Effect of the pH and the composition of the medium. Initially simple phosphate and veronal buffers were used in investigating the pH dependence of the HA titre. Since the results were often inconsistent completed buffers of

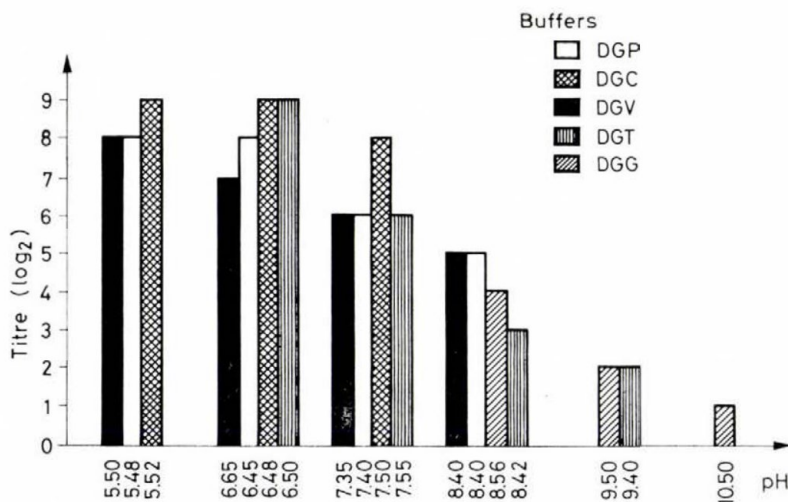


Fig. 1. pH dependence of the HA titre. Each column represents the mean for 4 to 8 parallel reactions. The difference between the parallels never exceeded 1 log₂ unit. Temperature 4 °C

0.025 M molarity were applied. Incomplete sedimentation, spontaneous HA and/or haemolysis commonly occurred at pHs below 5.5 when DGGI or DGC was used as medium. Only the range between pH 5.5 and 10.5 could be evaluated (Fig. 1). Within this range, the highest titres were obtained between pH 5.5 and 6.5; they were, however, influenced by the composition of the buffer, even at approximately the same pH. To eliminate these discrepancies, HSAG solution was introduced, a buffer widely used in rubella diagnostics [5, 11]. The titres obtained with this buffer at pH 5.75, 6.0 and 6.25 were identical with one another and with the highest titres shown in Fig. 1. Since the reactions developed in HSAG were the easiest to evaluate, in the following experiments this buffer was used exclusively.

An inverse relation was found between HA titre and NaCl concentration in the range 0.07 M to 2.570 M (Fig. 2).

Effect of incubation temperature. The HA reaction proved to be temperature-dependent, titres being inversely related to temperature (Fig. 3).

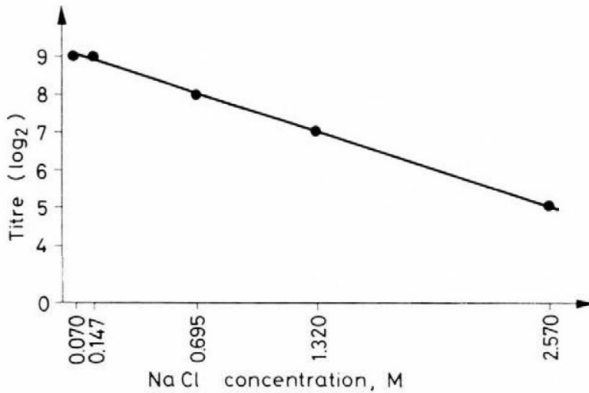


Fig. 2. Dependence of HA titre on NaCl concentration of the medium. The NaCl concentration was varied in HSAG buffers of pH 5.75 to 6.25

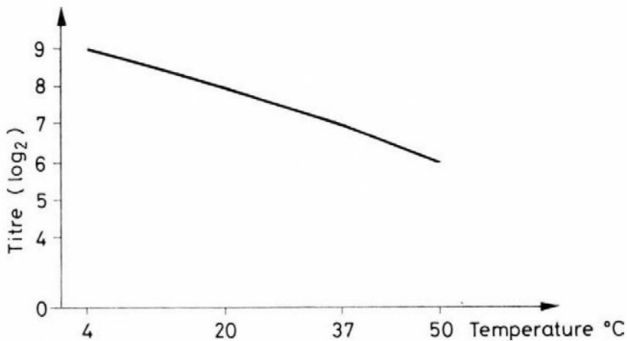


Fig. 3. Temperature dependence of the HA reaction. Medium: HSAG buffer pH 6.0

Resistance of the haemagglutinin

Temperature. Up to 37 °C, the titres were not influenced by temperature. Incubation at 56 °C was accompanied by a slow decline in titre; the rate of decline depended on the NaCl concentration (Fig. 4).

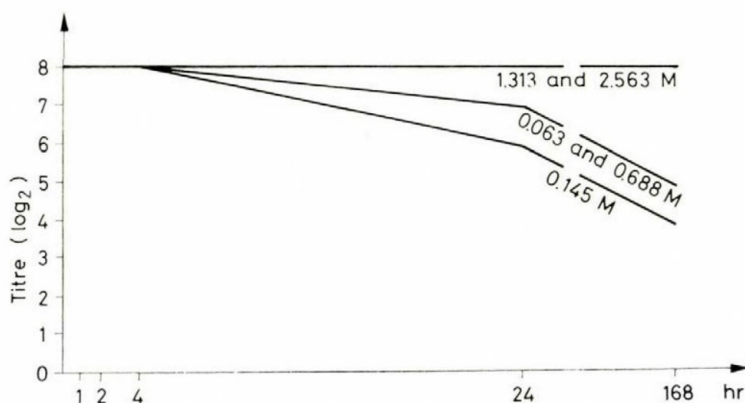


Fig. 4. Resistance of B.K. virus haemagglutinin to NaCl concentration. Preincubation at 56 °C, titration in HSAG buffer pH 6.0, at 4 °C

pH. Up to 37 °C, the HA titre was not reduced by the preincubation, independently of the pH of the preincubation medium and the length of the preincubation. A pH and time dependent decline was observed in the titre after a preincubation at 56 °C (Fig. 5).

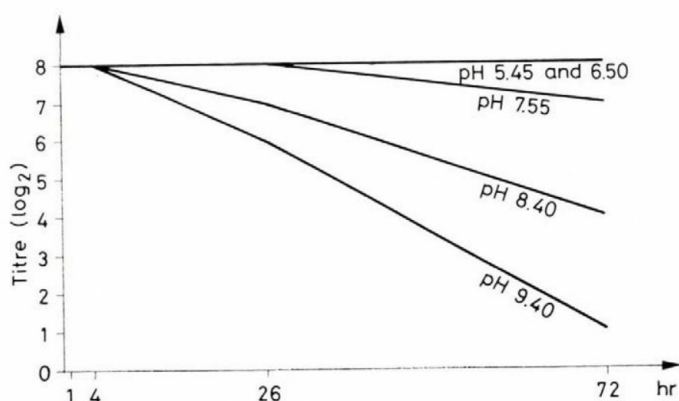


Fig. 5. pH resistance of the B.K. virus haemagglutinin. Preincubation at 56 °C, titration in HSAG buffer pH 6.0, at 4 °C

Discussion

We found that the HA reaction of B.K. virus haemagglutinin was pH-dependent. MÄNTYJÄRVI *et al.* [7] reported similar pH dependence, but they found the optimum in a slightly higher pH range. The difference might be attributed to the completed buffers used by us. MÄNTYJÄRVI *et al.* [7] used 0.05 M phosphate and veronal buffers in 0.15 M NaCl solution.

In citrate buffer, the results were somewhat different from those obtained in other media. It would be plausible to attribute the discrepancy to the lack of free Ca^{++} in the citrated medium, the more so that BRADY *et al.* [13] attributed a structure-stabilizing role to Ca^{++} in papovaviruses. We supposed, therefore, that in the absence of Ca^{++} B.K. virus particles may dissociate into haemagglutinating fragments, which might increase the HA titre. However, when we added 5 mM EDTA to simple or completed Tris buffer media (pH 6.5, 7.5 and 8.5) we observed no change in the HA titres.

As to the temperature dependence of the HA titre our results are consistent with those published by MÄNTYJÄRVI *et al.* [7], while TAKEMOTO and MULLARKEY [8] failed to observe any HA at 37 °C.

In our experiments, the optimum conditions for HA titration of B.K. virus were ensured by using HSAG buffer of pH 5.75 to 6.25 as medium, at an incubation temperature of 4 °C.

The resistance of B.K. virus haemagglutinin has been examined by TAKEMOTO and MULLARKEY [8] and PITKO *et al.* [12]. According to their results, the virion-associated haemagglutinin is resistant to ether, chloroform, 0.1% beta-propiolactone, and resists heat treatment at 56 °C for 30 min. To our best knowledge, nobody has reported on the pH and salt resistance of the B.K. haemagglutinin. It was shown, however, that dissociation of polyoma virus in a medium containing 50 mM ethylene glycol-bis-*N,N'*-tetra acetic acid, 3 mM dithiotreitol and NaCl solution of 0.2 to 0.6 molarity was inhibited by 1.0 M NaCl [13]. This seems to be in accordance with our observation that the B.K. virus haemagglutinin is stabilized in NaCl solutions of high molarity. It has also been reported that SV40 [14, 15] as well as human and bovine papilloma viruses [16] disintegrate in alkaline media. The pH resistance of our partially purified B.K. virus haemagglutinin agrees well with the reported resistance of various papovavirus haemagglutinins. An exact comparison to literary data is hindered by the different temperature and incubation circumstances.

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PHOTOCHROMOGENIC AND SCOTOCHROMOGENIC MYCOBACTERIA: THEIR CLINICAL SIGNIFICANCE

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In the period 1973–1977, *Mycobacterium tuberculosis* was isolated by cultivation in 4408 cases from the clinical specimens of patients with positive X-ray findings. On the basis of atypical colony morphology or pigment formation, 263 other mycobacterial strains were identified: of these 23 were photochromogenic and belonged to *Mycobacterium kansasii*. The strains were cultured on several occasions from the specimens of 4 patients with bronchopulmonary mycobacteriosis. The strains were resistant to isoniazid and streptomycin, sensitive to ethambutol and rifampicin. A total of 18 scotochromogenic isolates cultured from 14 patients with positive X-ray findings were identified as *Mycobacterium aquae* (*M. gordonae*) and its variants: strains showing slow Tween hydrolysis and 1 strain of rapid growth. In 5 cases *M. tuberculosis* was also obtained, indicating the presence of a mixed mycobacterial population. All scotochromogens were resistant to isoniazid and sensitive to ethambutol, with the exception of two strains sensitive to rifampicin.

During the past 20 years there has been special interest in human pulmonary diseases caused by so-called “atypical” mycobacteria. The term mycobacteriosis has been suggested for diseases produced by such infections, to differentiate them from tuberculosis. Mainly, photochromogenic mycobacteria of the Runyon Group I are the potential pathogenic agents [1].

The organism was isolated by BUHLER and POLLAK [2] in 1953 from patients suffering from disease resembling tuberculosis. The agent was later characterized by HAUDUROY [3], therefore at present the acid-fast species is named *Mycobacterium kansasii* Hauduroy. During the past two decades 536 cases were reported, where the pathogenic role of the organisms could be verified. GIMPL *et al.* [4–6] have devoted studies to the species in Hungary, as the disease occurs in Hungary from year to year.

The geographical distribution of mycobacteriosis is rather varied. Most of the cases were reported from the USA. Cases diagnosed at pulmonary wards range between 1 to 15%.

M. kansasii is an ubiquitous species. It can be demonstrated from plants, soil and products of animal origin. At present there is no direct evidence of its direct transmission from man to man. ONSTAD [7] described a familial occurrence in 1969.

Silicosis, anthracosis, immune deficiency states and haematological diseases are regarded as predisposing factors for the development of bronchopulmonary mycobacteriosis due to *M. kansasii*. Vigorous immune suppressive

and corticosteroid therapy are also worth mentioning with special regard to the hazard they represent for patients undergoing organ transplantation.

According to the Runyon classification, scotochromogens belong to Group II of atypical mycobacteria. The strains grow slowly and produce yellow or orange colonies even when grown in dark. The group is heterogeneous, identification of the strains presents difficulties. Several acid-fast species with a variety of names belong to the group, such as *Mycobacterium scrofulaceum* (*M. marianum*) [8], *Mycobacterium flavescens* and *Mycobacterium gordonae* [9]. The latter is most probably identical with *Mycobacterium aquae* [10]. The strains can be divided into three subgroups on the basis of their amidase spectrum [11, 12]. WAYNE *et al.* [13] distinguished two subgroups among the scotochromogens, a Tween 80 hydrolyzing and a non-hydrolyzing group. The antigenic structure of scotochromogenic strains was studied by CASTELNUOVO and MORELLINI [14]; they demonstrated a similarity between *M. scrofulaceum* and *M. aquae*. GIMPL [15] arrived to the same results when assuming the existence of subgroups. According to WAYNE *et al.* [13], the two species can be differentiated by immunodiffusion. MAGNUSSON *et al.* [16] could distinguish two subgroups in examinations using sensitin. Three subgroups were determined by SCHAEFER [17] with the help of agglutination.

Since scotochromogenic mycobacteria are ubiquitous species, they may be present in tap-water, too. In spite of this, several data indicate their pathogenic role [18–21]. Joos *et al.* [22] reported on a child with generalized scotochromogenic mycobacteriosis, GIMPL *et al.* [23] described bronchopulmonary mycobacteriosis in a patient suffering from rheumatoid arthritis.

In the period 1973–1977, 23 photochromogenic and 18 acid-fast scotochromogenic strains were studied by microbiological and biochemical methods and identified in order to clarify their possible pathogenic role. The photochromogens were cultured from specimens of 4 patients who had positive X-ray findings and tuberculosis-like disease, the scotochromogens from specimens of 14 patients also positive roentgenologically. The cellular and humoral immunity of the patients with mycobacteriosis was also examined.

Materials and methods

Mycobacterium strains. Of the identified 263 species 23 were photochromogenic; from among the scotochromogens 18 strains were selected. Sputum specimens were pretreated with N NaOH and inoculated on Löwenstein–Jensen and Sula media. The strains to be identified were then transferred to Löwenstein–Jensen medium every two weeks.

Bacteriological methods. From the Löwenstein–Jensen medium, two-week-old culture suspensions diluted 10^5 and the isolated colonies producing pigment on light stimulation or when grown in dark were subcultured. When a homogeneous culture had been obtained, a suspension diluted 10^{-5} was prepared from the 2-week-old medium and of this 0.1 ml was inoculated. (a) Growth was read after incubation at 22–25 °C, 33–39 °C and 41–43 °C [24]. The strains appearing within 7 days were regarded as rapidly growing, those after 7 days as

slowly growing. (b) Proportional indirect antibiograms were made after CANETTI *et al.* [25]. (c) Pigment production in dark and on light exposure was examined.

Biochemical methods. The following tests were carried out: (a) niacin reaction; (b) catalase reaction; (c) examination of amide series [11]; (d) nitrate reductase activity [26]; (e) iron utilization [27]; (f) tween hydrolysis [26] at 5 and 10 days.

Large intraperitoneal or intravenous doses of photochromogenic mycobacteria produce regressive lesions in the mouse, the golden hamster and the guinea pig. In the guinea pig the effect is enhanced if *M. kansasii* is inoculated intratracheally and quartz crystals are introduced into the lung.

Scotochromogenic mycobacteria (Runyon Group II) are practically non-pathogenic for experimental animals [28], nevertheless they might have some pathogenicity for humans. They might live and multiply in the human organism, a fact supported by the great number of scotochromogenic PPD reactors. Some authors maintain that the tonsils are the reservoirs of scotochromogenic mycobacteria. FROMAN examined specimens obtained from 107 routine tonsillectomies and could isolate 6 scotochromogenic species (cit. ONSTAD [7]).

Using the methods listed before, we cultured beside the 4408 *M. tuberculosis* strains 23 photochromogens from specimens of four patients and 18 scotochromogens from specimens of 14 patients. In case of the photochromogenic strains no other mycobacteria could be demonstrated, thus repeated identification implies infection with *M. kansasii*. With the scotochromogens, *M. tuberculosis* was cultured on five occasions. The diagnosis at discharge was pulmonary tuberculosis in 5 patients, bronchopneumonia-pleuropneumonia in 5, chronic polyarthritis in 1 and lung tumour in 1 case. From one patient with large cavities, *M. tuberculosis* and a scotochromogenic mycobacterium was isolated on five occasions.

Bronchopulmonary mycobacterioses. Primary and secondary criteria may be of help in the diagnosis [29]. The most important are repeated cultivations with large numbers of bacteria and the fact that no *M. tuberculosis* should grow in the culture. At the same time the X-ray findings and the clinical picture indicate a disease resembling tuberculosis.

Photochromogenic mycobacteria are slightly virulent for experimental animals; in spite of this, they produce tuberculosis-like X-ray findings and disease in man. Mainly, *M. kansasii* can be demonstrated as the pathogenic agent.

Mixed mycobacterioses. The criterion of infection is the repeated cultivation of *M. tuberculosis* and that of other *Mycobacterium* species [30, 21]. *M. tuberculosis* + *M. avium*, *M. tuberculosis* + *M. kansasii*, *M. tuberculosis* + *M. aquae* were cultured from human isolates. Pneumoconiosis was found to be a predisposing factor.

Photo- and scotochromogenic mycobacteria are non-pathogenic for experimental animals. Literary data indicate that mycobacterioses occur mainly in subjects with immune deficiency. Therefore, we examined the humoral and cellular immunity of 2 patients with mycobacteriosis and of 2 with mixed mycobacteriosis, by immunoelectrophoresis [31], and lymphocyte marker examination.

Results

Photochromogenic mycobacteria occur in nature, but the mode of human transmission is unclear. If they can be demonstrated from patients with pulmonary lesions, the condition is termed mycobacteriosis. Such cases are registered every year.

The isolated strains can be cultured at room temperature and at 37 °C but not at higher temperatures. Niacin negativity and catalase positivity are characteristic properties as also their intensive Tween hydrolysis, urea and nicotinamide decomposition and nitrate reductase positivity. If there is pigment production on light exposure, the strains can readily be identified (Table I). The strains are resistant to isoniazid and streptomycin, sensitive to ethambutol and rifampicin.

The tuberculosis-like pulmonary lesions are characterized by early cavity formation (Figs 1 and 2). In the past, these patients had to be operated upon;

Table I
Properties of the cultured photochromogenic mycobacteria

No. of patients	4
No. of strains	23
Growth	
slow	23
22–25 °C	23
33–39 °C	23
41–43 °C	0
Niacin	–23
Catalase	+23
Nitrate reductase	+23
Urea, nicotinamide	+23
Tween hydrolysis (5 days)	+23
Iron utilization	–23
X-ray changes in the lung	4

at present bacterium excretion ceases on the administration of ethambutol and rifampicin, the patients become symptom-free and the cavity disappears.

Two patients examined by immunoelectrophoresis had increased IgG levels. Quantitative data of T and B lymphocytes were practically normal.

The scotochromogenic strains grew at room temperature and at 37 °C. A rapidly growing strain grew also at 41–43 °C. Nine strains hydrolyzed Tween of the 5th, 17 strains on the 10th day, 1 strain obtained from a patient with

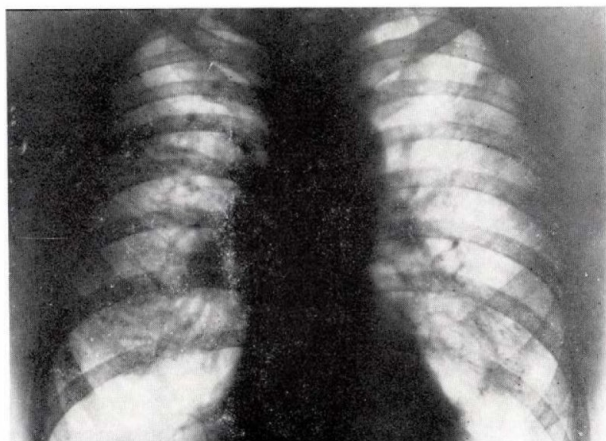


Fig. 1. X-rays of bronchopulmonary photochromogenic mycobacteriosis

mixed mycobacteriosis, after 10 days. Nitrate reductase activity was negative in part of the strains, but in a number the test was doubtful when repeated on several occasions. Three groups could be distinguished by amide series. One strain which failed to decompose amides, corresponded to non-urealytic *M. aquae* (*M. gordonae*) referred to by BÖNICKE. Urea was decomposed by 17, nicotinamide and pyrazinamide by 2 strains. Of these, 1 rapidly growing and



Fig. 2. Tomography of bronchopulmonary photochromogenic mycobacteriosis

iron utilizing strain was characterized as *Mycobacterium phlei*. This strain, too, was isolated from a case of mixed mycobacteriosis. Microbiological and biochemical properties of the identified scotochromogenic strains are shown in Table II. All these patients had positive X-ray findings. The scotochromogenic strains were resistant to isoniazid, susceptible to ethambutol and, except 2 strains, to rifampicin.

In 5 cases scotochromogenic mycobacteria were cultured together with *M. tuberculosis*. Scotochromogenic strains were cultured on one occasion each in 13 cases, on five occasions in 1 case. In the latter, *M. tuberculosis*, too, was demonstrated.

The majority of patients had displayed X-ray changes for years. All but 1 patient received regularly antituberculars, a number also antibiotics and corticosteroids. In patients with mixed mycobacteriosis gross radiologic lesions

Table II
Properties of the cultured mycobacteria

No. of patients	14
No. of cultured strains	18
Scotochromogenic	18
Growth	
rapid	1
slow	17
22-25 °C	+18
33-39 °C	+18
41-43 °C	+ 1
Niacin	0
Catalase	+18
Urea	+17
	- 1
Urea, nicotinamide, pyrazinamide	+ 2
Iron utilization	-17
	+ 1
Tween hydrolysis (5 days)	+19
Tween hydrolysis (10 days)	+18
Nitrate reductase	±18
X-ray changes in the lung	14

were observed. Bacterium excretion ceased and the lesions regressed on adequate treatment (Figs 3 and 4).

Immunoelectrophoresis showed the increase of IgG and IgM in two patients with mixed mycobacteriosis (Fig. 5); T and B lymphocyte conditions were normal.

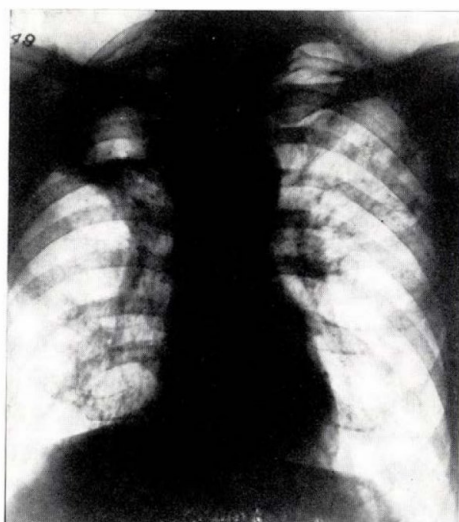


Fig. 3. X-rays of mixed mycobacteriosis, *M. tuberculosis* + scotochromogenic strain



Fig. 4. Tomography of mixed mycobacteriosis in right lung. Large cavity

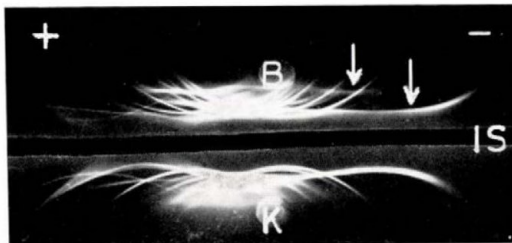


Fig. 5. Immunoelectrophoretic pattern of the serum of a patient with mixed mycobacteriosis. Increase of IgG and IgM. C = control serum, P = patient serum, IS = anti-human horse serum

Discussion

A small number of bronchopulmonary photochromogenic mycobacterioses have been revealed in Hungary, and more are expected to occur in the future in view of the more extensive application of corticosteroid and immune suppressive therapy. In our patients complete recovery could be achieved by rifampicin and ethambutol treatment; development of the disease seemed to be in no connection with the humoral and cellular immune state of the patients.

Scotochromogenic mycobacteria could be demonstrated from patients with X-ray changes. Under the conditions existing in Hungary, the occurrence of mixed mycobacterioses is of special interest. We could not prove the patho-

genic role of scotochromogenic bacteria, nevertheless it may be assumed that their presence aggravates the condition of the affected lung and hinders recovery. Inactive or active tuberculosis may also have a role in the development of mixed mycobacteriosis. No connection could be found between the immune status and the disease, but all chronic pulmonary processes may facilitate the colonization of scotochromogenic mycobacteria in the lung. Our studies indicate that ethambutol and rifampicin therapy is partly effective against scotochromogenic mycobacteria, too.

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A RHABDOVIRUS ISOLATED FROM CARPS IN HUNGARY. EXPERIMENTAL INFECTION OF CARPS AND RESISTANCE OF THE VIRUS

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(Received March 17, 1978)

From carps showing the symptoms of acute infectious dropsy, a virus was isolated for the first time in Hungary. On the basis of physicochemical and serological tests and electron microscopy, the virus was identified as spring viraemia carp virus and assumed to have a primary role in the acute form of infectious dropsy known so far as a bacterial disease.

The role of some virus in the acute dropsy of carp had been assumed by many authors [1] before the virus (*Rhabdovirus carpio*) was first isolated in Yugoslavia by FIJAN *et al.* [2] in 1971. Of the carps artificially infected with the virus, 90% died with characteristic symptoms and the virus could be re-isolated in every case. Antibiotic treatment failed to reduce the mortality. The disease caused by *R. carpio* was termed spring viraemia of carp by the Yugoslav authors.

Soviet authors had reported the isolation of cytopathogenic (CP) agents which were later identified as *Rhabdovirus* [3]. Isolation of a similar virus has been reported from Czechoslovakia [4], the G.F.R. [5] and France [6]. Other investigators have studied the morphology [7] and the serological properties [8] of *R. carpio* in comparison to those of the *Rhabdovirus* family.

In Hungary, viral aetiology of fish diseases could not be studied until recently, when preparation of fish cell cultures was initiated [9] and several widely-used cell lines were imported.

Materials and methods

In the spring of 1976, virus isolation was attempted from all the carps submitted to our Institute with symptoms of acute dropsy. Homogenates of different organs were centrifuged to reduce bacterial count. The supernatants were inoculated into tube cultures of the EPC cell line. This and other cell lines as well as the prototype *R. carpio* strain RC had been kindly supplied by Dr. N. FIJAN (Zagreb, Yugoslavia). The cultures were incubated at 18–20 °C and examined for CP changes daily. For this purpose preparations were embedded in celloidin and stained with haematoxylin–eosin.

Isolates were also inoculated into FHM, BB and RTG-2 fish cell lines as well as into primary cultures of carp ovary cells. Calf testicle and lamb kidney tissue cultures and the calf kidney cell line MDBK inoculated with the isolates were incubated at 37 °C and 20 °C.

Several physicochemical properties of an isolate (T-13) were determined and compared to those of the prototype *R. carpio* (RC) strain. Plaque-reduction tests [10] were performed

with a *Rhabdovirus* antiserum prepared in this Institute and a reference serum kindly supplied by Dr. J. B. HILL (Dorset, England).

For experimental infection, carps of 150 g body weight free from dropsy were obtained from a fishery. We established 9 experimental groups to investigate virus infectivity (Table I). During the experiment water temperature was kept at $15 \pm 3^\circ\text{C}$.

Spontaneously died specimens were subjected to bacteriological tests.

The virus propagated in the EPC cell line was examined in negative contrast preparations electron microscopically.

Resistance of the strain T-13 was investigated *in vitro*. The virus was exposed to pH 3, various temperatures and freeze-drying. It was also exposed to disinfectants, *viz.* 0.5 and 1.0% Iosan (Phylaxia, Budapest) and 3% and 5% formalin. After an incubation with disinfectant at 18°C for 5 or 10 min, the virus was immediately diluted and titrated.

Results

The first CP agent was isolated when death of carps was observed in pond K. of anglers' club T. Moribund carps swimming near the shore could be caught by hand. They appeared darker than usual and haemorrhages were seen on their skin. The gills were pale and striped, moderate exophthalmus, and haemorrhages on the bottom of the anterior eye chamber were observed. The mild abdominal hydrops soon became pronounced and the anus protruded. At postmortem examination serous-fibrinous peritonitis, catarrhal-haemorrhagic enteritis, and oedematous swelling of organs were seen. Haemorrhages occurred in various organs, first of all in the wall of the swim-bladder and in the pericardium, further, in the mucosa covering the posterior wall of the branchial cavity and in the gills. Serous exudate was found in the cranium, accompanied by dilatation and haemorrhages of meningeal vessels.

Further investigations were performed with the first isolate called T-13. When incubated at $18\text{--}20^\circ\text{C}$, the inoculated EPC cells showed CP changes after 30–40 hr. Cells rounded off and detached from the glass wall. Stained preparations showed cell degeneration, cumulation of chromatin at the nuclear membrane and chromatin granulation; the nuclear membrane was loosened and pale and soon disappeared (Fig. 1). We failed to find inclusions. In cell lines FHM, BB and RTG-2, well-defined CP effect appeared within 48–72 hr. Of the cell lines RTG-2 proved to be the least sensitive. The primary cell cultures of carp ovary responded like the EPC line. Mammalian cell cultures, even the non-infected ones, degenerated at $18\text{--}20^\circ\text{C}$ and proved therefore unsuitable for culturing the virus.

The T-13 isolate as well as the RC strain used as control, proved sensitive to 56°C , pH 3, and chloroform; replication was not inhibited by IUdR.

Electron micrographs showed enveloped bullet forms about 140 nm in average diameter, characteristic of *Rhabdovirus* (Fig. 2).

Plaque-reduction assay was performed with the $10^{-3.5}$ dilution of the virus and read on the 7th day after inoculation. The CP effect of the T-13 isolate was inhibited by both sera.

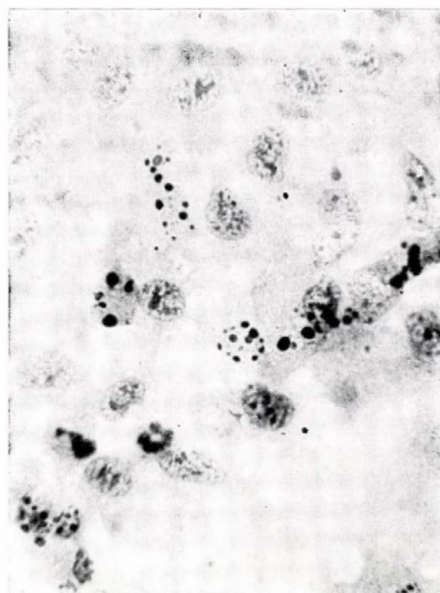


Fig. 1. CP effect of T-13 virus strain in EPC cell culture. Haematoxylin and eosin, $\times 1400$



Fig. 2. Electron micrograph of *Rhabdovirus* (T-13). Negative contrast, $\times 360\ 000$

Table I
Experimental infection of carps with the isolate T-13

Group no.	Treatment	Number of animals	
		died/treated	died/control
1	T-13 10^{-1} 0.2 ml i.p.	2/4	0/2
2	T-13 10^{-2} 0.2 ml i.p.	2/4	0/2
3	T-13 10^{-3} 0.2 ml i.p.	2/4	0/2
4	T-13 10^{-4} 0.2 ml i.p.	4/4	0/2
5	Virus-contaminated Tubifex p.o.	1/4	—
6	T-13 10^{-1} 0.5 ml p.o.	1/4	—
7	T-13 10^{-1} 0.5 ml p.o. + glass powder	2/4	—
8	Glass powder p.o.	0/4	—
9	T-13 10^{-2} ml i.p. + 50 mg/litre chloramphenicol	3/4	0/2

i.p. = intraperitoneally

p.o. = orally

Table I shows the results of experimental infections carried out with the T-13 isolate. Half of the carps inoculated intraperitoneally with the 10^{-1} to 10^{-3} dilutions of the virus survived the disease, while the four fish inoculated with a 10^{-4} dilution died. Oral inoculation with a large dose was not always successful as reported also by FIJAN *et al.* [2]. This seemed to be supported by simultaneous administration of powdered glass, suggesting that intestinal damage may predispose fishes to fatal disease.

Implantation of virus introduced by food animals suggested a possible role of lower organisms in the infection. Simultaneous administration of chloramphenicol failed to prevent fatal infection. Furthermore, we failed to prove any role of sick fish as the source of infection, *viz.* none of the contact controls contracted the disease. The virus was consistently re-isolated from the organs of died fish and bacteria belonging to the genus *Aeromonas* were isolated from the kidneys.

The disinfectant dilutions used destroyed the CP activity of the T-13 isolate within 5 min. The virus lost almost its entire activity within 30 min at pH 3 and within 15 min at 45 °C. Its infective titre was hardly reduced during 6-month storage at -30 °C or in the freeze-dried state.

Discussion

The dropsy of carp observed in Hungary was attributed to *Aeromonas punctata* by BUZA and SZAKOLCZAI in 1968 [1]. Since then, however, a virus has been consistently isolated from carps with acute dropsy (2-5) and the virus

proved to cause similar symptoms in artificially infected carps [2]. Thus, the virus might therefore play a primary role in the aetiology of the disease.

Recently, the designation "spring viraemia of carp" proposed by FIJAN [11] has become accepted in the literature in order to differentiate the acute form of infectious dropsy of carp from the subacute and chronic forms. The same author has recommended the term "carp erythrodermatitis" for the subacute and chronic forms which, in fact, represent a bacterial disease [12]. By the use of this designation, the confusing name dropsy may be avoided.

We have proved that the T-13 strain, i.e. the representative of the strains isolated by us, corresponds in properties to the prototype strain of *R. carpio*. Experimental infections have shown that in the carps died with characteristic symptoms of acute disease, bacteria belonging to the genus *Aeromonas* were present besides the virus.

Some role of predisposing factors may be assumed, since several carps infected by *Bothriocephalus* were the first to die and virus implantation seemed to be supported by artificial intestinal damages.

The failure of preventive antibiotic (chloramphenicol) treatment suggests that the antibiotic-containing food often recommended for the therapy of acute dropsy cannot be efficient against spring viraemia of carp.

The virus proved to tolerate cold and be sensitive to disinfectants, e.g. Iosan (Phylaxia, Budapest), which in 0.5% solution inactivates the virus within 5 min. Iosan is suitable for disinfecting instruments and equipment.

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POSTEPIDEMIC SPREADING BY WILD BIRDS OF THE INFLUENZA A VIRUS VARIANT VICTORIA/75

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One year after the influenza epidemic A/Victoria/75 had subsided in Hungary, 46% of captive birds in a zoo were found to possess serum antibodies to this variant. Of the avian influenza virus subtypes, only Hav7 accounted for seropositivity in 24% of the zoo bird population, exclusively in anatid or anserine species. Examination of the eggs laid by migrating wild birds which had arrived to the country some weeks before the zoo investigation, revealed the presence of yolk antibodies to subtype Hav3 in 8.9% of gull eggs, to subtype Hav5 in 2.1–4% of tern and wild duck eggs, and to subtype Hav7 in 4.0, 7.4 and 17.9% of wild duck, tern and gull eggs, respectively. Haemagglutination-inhibiting yolk antibodies to Victoria/75 were detected exclusively in 7.8 and 13.8% in gull and tern eggs, respectively, but were still found in the second batch of eggs laid 6 weeks later, and young birds which by that time had hatched from the first-laid eggs had yolk-derived serum antibodies to Hav7 and Victoria/75 at a proportion similar to yolk positivity. Among the wild birds shot for investigation, Hav7 virus was isolated from one gull and one tern, whereas Victoria/75 virus from another gull; a subtype Hav6 strain was isolated from a starling shot elsewhere in a wild bird reservation area.

Spread of the influenza virus variant A/H3N2/Victoria/75 among zoo birds as well as resident and migrating wild birds simultaneously with, or shortly after, the appearance of the epidemic in Hungary has been reported [1, 2]. Seropositive wild birds were detected soon after involvement of the population by the epidemic in each geographical region studied, and their proportion usually rose as the outbreak was progressing. Evidence was presented that the zoo birds, kept during the winter in an isolated bird house, contracted the infection from attendants and visitors. Isolation of a strain corresponding to Victoria/75 from a sparrow captured near the river Tisza, and of the earlier variant, England/72, from a turtle dove captured at the zoo bird pond, Budapest, have also confirmed the spread of human influenza virus to wild birds [3, 4]. Evidence has, however, been lacking of the postepidemic persistence of the variant Victoria/75 in wild bird populations in different geographical areas.

Materials and methods

Virus isolation was attempted from a total of 67 wild birds resident in, or migrating through, a pond farm and two bird reservation areas situated between the rivers Danube and Tisza, and near the town Szeged, respectively. Tracheal and cloacal swabs were collected from wild birds shot in these areas from March to June, 1977, and placed in phosphate-buffered

saline (pH 7.2) containing penicillin, streptomycin and kanamycin at 4 °C, and 0.1 ml volumes were inoculated within 24 hr into the amniotic and allantoic cavity of 11-day-old chick embryos. The eggs were incubated at 35 °C for 48–96 hr and tested individually for haemagglutinin activity.

Virus strains. The A/H3N2/Victoria/3/75 reference strain, the avian influenza virus subtype Hav1–Hav7 reference strains, and antisera were used for identification of the isolates in the haemagglutination-inhibition (HI) test.

Serum and yolk samples. Serum samples were collected in a provincial zoo from birds of 17 species, above all anatid and anserid birds, pheasants and storks, but also from cormorants, ravens and some other species. The yolks of eggs laid by migrating birds (mallards, gulls and terns) and sera of newly-hatched, 1–3 days old progeny, were also included in the serological screening.

Serological tests. The HI tests were performed according to the Standard Prescriptions of the WHO Expert Committee on Respiratory Virus Diseases [5]. The test sera were inactivated at 56 °C for 30 min and were subsequently treated with KIO₄ and, occasionally, also with *Vibrio cholerae* filtrate (RDE, Wellcome Reagents Ltd., London). Yolk antibody assays were carried out as proposed by CHU and BARHOUMA [6].

Results

The epidemic caused by the Victoria/75 strain had subsided in Hungary by late spring in 1976, but sporadic cases, verified by virus isolation, continued to occur. In 1977, blood samples were taken at random from 50 captive

Table I

Zoo birds possessing haemagglutination-inhibiting antibodies to influenza virus A/H3N2/Victoria/75 and to the avian influenza virus subtypes Hav1–Hav7

Species	Date of sampling	No. of samples	No. of samples positive to			HI antibody titres to	
			variant	subtype		Victoria/75	Hav7Neq2
				Victoria/75	Hav7	Hav1–Hav6	
House duck	15. 4.	4**	2	3	—	1 : 16–1 : 32	1 : 24–1 : 64
Mallard	1977	7	3	1	—	1 : 16 (3)***	1 : 24
Mute duck		3**	3	2	—	1 : 16–1 : 32	1 : 24–1 : 64
Shelduck		2	—	1	—		1 : 128
Ruddy Shelduck		2	—	2	—	—	1 : 24 (2)
House goose		2*	1	2	—	1 : 16	1 : 24–1 : 32
Bean goose		1*	1	1	—	1 : 16	1 : 32
Pheasant		7	6	—	—	1 : 16–1 : 64 1 : 128(3)	—
White stork		7	4	—	—	1 : 16–1 : 32	—
Cormorant		3	2	—	—	1 : 16 (2)	—
Greater flamingo		1	1	—	—	1 : 32	—

* and ** One and two birds, respectively, possessed serum antibodies to both Victoria/75 and the Hav7 reference strain

*** The figure in parentheses indicates sera with identical HI titre

birds of 17 species in a zoo situated in a forest near a provincial town, for examination for antibodies to Victoria/75 and to the seven avian influenza virus subtypes (Hav1–Hav7) available in this laboratory. Antibodies to Victoria/75 were found in 23 (46%) samples; most frequently in sera from pheasants, anatid birds and storks. Among the avian subtypes, HI antibodies were found only to Hav7. The positive birds numbered 12 (24%) and all were either anatid or anserine species (Table I). Some serum samples contained antibodies to both Victoria/75 and Hav7.

In the same period, 5 (16.1%) of 31 slaughtery turkeys, 2–2.5 years old, and 7 (31.8%) of 22 one to two years old pheasants reared in captivity, were found to possess HI antibodies to Victoria/75.

Three weeks after the zoo investigation the eggs laid by migrating wild birds — common tern (*Sterna hirundo*), black-headed gull (*Larus ridibundus*), little gull (*Larus minutus*), mallard (*Anas platyrhynchos*), pochard (*Aythya ferina*) and ferruginous duck (*Aythya nyroca*) — in the area of a pond farm were examined for yolk antibodies to avian influenza viruses and Victoria/75. Among the eggs of black-headed gulls 8.9% contained antibodies to subtype Hav3, while the yolks of several eggs laid by anatid birds, little gulls and common terns contained HI antibodies to Hav5 in low per cent (Table II). We were surprised to find HI antibodies to Victoria/75 in gull and tern and to subtype Hav7 in wild duck, tern and gull eggs in a high proportion of the examined yolks. Such antibodies were also detected in eggs collected from black-headed gulls in an agricultural farm near the river Tisza.

At the survey of wild bird eggs layed in the pond farm 46 days after the first sampling, yolk antibodies to Victoria/75 and Hav7 were found in roughly equal proportions in gull and tern eggs.

After hatching of young birds, serum samples were collected for antibody assay at a few days or weeks of age. Among 21 young terns 4 (19.0%) and among 11 gull chicks one (9.0%) had serum antibodies to Victoria/75, while the serum of another single tern (4.7%) reacted with the Hav7 virus.

Among the wild birds — mallard duck, pochard, black-headed gull, coot, common tern, redshank (*Tringa totanus*), black tern (*Chlidonias niger*) — shot in the pond farm area in May and June, 1977, during egg collection, influenza virus was isolated from three; subtype Hav7 virus was isolated from the sinus of one gull and the cloacal mucosa of one tern, while Victoria/75 from the rinsing fluid obtained from the infraorbital cavity of another gull. Subsequent isolation experiments — lapwing (*Vanellus vanellus*), common sandpiper (*Tringa hypoleucos*), common tern, starling (*Sturnus vulgaris*) and coot — in a wild bird reservation area situated between the rivers Danube and Tisza resulted in isolation of a subtype Hav6 strain from the upper respiratory mucosa of one starling.

Table II

Haemagglutination-inhibiting yolk antibodies to avian influenza virus subtypes and Victoria/75 in eggs laid by different wild birds

Total number of eggs, 293

Bird species	Date of egg collection	No. of eggs	HI antibodies in yolk to			
			Avian influenza virus subtypes			Variant Victoria/75
			Hav3	Hav5	Hav7	
<i>Pond farm</i>						
Mallard, pochard, ferruginous duck	6. 5. 1977	25	—	1 (4%) (1 : 40–1 : 80)	1 (4%) (1 : 20–1 : 40)	—
Black-headed gull		89	8* (9%) (1 : 20–1 : 40)	—	16** (18%) (1 : 10–1 : 40)	7*** (8%) (1 : 20–1 : 40)
Little gull		5	—	2 (1 : 40)	—	—
Common tern		94	—	2 (2%) (1 : 20–1 : 80)	7 (7%) (1 : 10–1 : 40)	13 (14%) (1 : 20–1 : 160)
<i>Agricultural farm</i>						
Black-headed gull	28. 4. 1977	5	—	—	4 (1 : 20)	—
<i>Pond farm</i> ⁺						
Black-headed gull	21. 6. 1977	35	—	—	4 (11%) (1 : 20–1 : 160)	3 (9%) (1 : 20–1 : 80)
Common tern		40	—	—	3 (8%) (1 : 10–1 : 20)	4 (10%)

* Two gulls with yolk antibodies to Victoria/75

** Two gulls with yolk antibodies to Hav3 subtype

*** Three gulls with yolk antibodies to Victoria/75 and Hav7

+ Eggs collected in the same laying area 46 days later

Discussion

The Victoria/75 epidemic had spread to Hungary early in 1976 and subsided by springtime, but sporadic infections verified by virus isolation continued to occur sporadically also later in that year. Spread of the infection to wild birds was demonstrated both directly and indirectly [1] during the epidemic period, but we were surprised to detect one year later antibodies to Victoria/75 in a considerably high proportion of captive birds kept in a provincial zoo, separated from its surroundings by a wire mesh fence. Subsequent detection of haemagglutination-inhibiting yolk antibodies to Victoria/75 in eggs laid by gulls and terns in the area of a pond farm, and isolation of Victoria/75 virus from migrating gulls were indicative of the persistence of the agent in certain populations of both wild bird species. The migrating gulls and terns had either become infected during the previous year's epidemic and continued to shed the virus among their populations, or during later sporadic occurrences of the disease among humans, which may in principle also have been responsible for the infection of the isolated zoo birds.

In winter 1976-1977 the gulls indigenous in Hungary migrated to the southern part of Europe and to the Near East, while the terns migrated *via* the Balcan, eastern littoral of the Mediterranean Sea and Egypt to Central and Southern Africa, even as far as Moçambique, as reported by the Ornithology Institute, Budapest. According to the WHO reports, disease due to the variant Victoria/75 did occur among humans in the transit or target areas of wild bird migration; thus, early in 1977 in Turkey, in February, 1977, in Bulgaria and Italy, in March in Egypt, Israel and Spain, and in April in Yugoslavia. The gulls and terns seeking rest in these countries during their return migration in early spring may well have contracted the infection during transit. The virus Victoria/75 may have persisted long among the birds, to judge from the presence of yolk antibodies in eggs freshly laid by gulls and terns in the peat marsh islets belonging to a pond farm. Yolk antibodies were present in a similar proportion of eggs, and at similar titres at the first and second egg layings separated by 46 days. By the time of the second laying the eggs produced on first instance were either in an advanced stage of embryonation or had already hatched. Part of the newly-hatched or very young gull and tern chicks possessed yolk-derived serum antibodies at titres roughly equal to those found in yolks.

The antibodies in the zoo birds may have been due either to infection during the epidemic and subsequent re-exposure(s) to sporadic human cases, or to fresh infection through contact with free-living migrating birds.

Another remarkable issue of the present studies has been the detection of HI antibodies to subtype Hav7 of avian influenza virus in the serum or yolk of a substantial proportion of wild birds and zoo birds, and the isolation of Hav7 virus from gull and a tern shot down for investigation along with

other wild birds. Of the avian influenza virus subtypes only Hav7 was demonstrable in zoo birds, while among the migrating wild birds 2–9% had antibodies to Hav5 and Hav3, too. Infection by strains of the latter two subtypes had presumably occurred during northward migration. Only few bird serum samples had contained HI antibodies to both Victoria/75 and Hav7, even to Victoria/75 and Hav3, respectively. In these cases the possibility of recombination of the two strains cannot be ruled out.

After onset of the A/Hong Kong pandemic in 1968, it was found [7] that the variant HK/68(H3N2), the duck isolate Hav7Neq2 obtained in the Ukraine in 1963, and the strain Heq2Neq2, isolated from horses in Miami, USA, also in 1963, had haemagglutinin antigenic component (HA2) in common; this finding was retrospectively confirmed by LAVER and WEBSTER [8]. During recent years, especially since the epidemic due to Victoria/75, a substantial part of avian influenza virus isolates obtained from birds in different parts of the world was identified as subtype Hav7. E.g. approximately 50% of the isolates deriving from wild birds indigenous in the far eastern territories of the USSR belonged to subtype Hav7, and one fourth of the avian influenza virus strains isolated from chickens, geese and, above all, ducks in the Hong Kong poultry abattoir in 1976 was of this type [9]. The abattoir isolates showed two orders lower HI titres with Victoria/75 than with homologous isolates or with the Hav7 found to be fairly frequent also in Hungary, but not so much among the domestic poultry as among zoo birds and migrating wild birds. In the bird populations studied by us, the proportion of infection by Hav7 and Victoria/75 was roughly similar. This and the presence of common haemagglutinin components in Victoria/75 and Hav7 strains support the assumption that the latter avian subtype strains, especially those possessing neuraminidase N2, may have developed from the virus variant A/Victoria/75 through adaptation in the course of bird passages. Another information emerging from the present studies is that the migrating wild birds may have contracted infection by avian influenza virus strains and by the A/Hong Kong variant Victoria/75 as well, either during their migration or during transitory residence in this country, and may well have transmitted it by contact to domestic and zoo birds.

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STREPTOMYCIN SENSITIVITY OF RIBOSOMES ISOLATED FROM A STREPTOMYCIN-PRODUCING STREPTOMYCES GRISEUS

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The streptomycin sensitivity of ribosomes derived from a streptomycin-producing *Streptomyces griseus* was examined in a polyuridylic acid directed ^{14}C -phenylalanine incorporating system. In order to get reproducible results it is essential to use cell-free extracts which do not inactivate streptomycin. This condition can be fulfilled by the combination of washed ribosomes of the streptomycin-producing strain and the 110 000 g supernatant of the streptomycin-nonproducing variant of *S. griseus*, because the streptomycin-phosphorylating activity can be washed out from ribosomes of younger streptomycin-producing cultures, and the streptomycin-nonproducing *S. griseus* does not have any streptomycin-inactivating capacity. In this amino acid polymerizing system the ribosomes of the streptomycin-producing strain were as sensitive to streptomycin as the ribosomes of the nonproducing variant or of *Escherichia coli*.

All streptomycin (SM)-producing strains contain SM-inactivating enzyme(s) [1–6], though OGAWA and PERLMAN [7] could obtain a SM-producing variant from a nonproducing *Streptomyces griseus* with the aid of an actinophage which was unable to inactivate SM by phosphorylation. We have also found intracellular SM-inactivating capacity in our *S. griseus* strain (No. 52-1) even at the quasi exponential stage of its life-cycle, when no SM could be detected in the culture broth by bioassay. This SM-inactivating mechanism makes it difficult to study the SM sensitivity of ribosomes derived from the SM-producing strain [8]. In our amino acid incorporating experiments using ribosomes from the SM-producing strain, freed from SM-phosphorylating activity, and the S-100 fraction (110 000 g supernatant) from the non SM-producing *S. griseus*, that is also inactive in SM-phosphorylation, we have a “pure” *in vitro* system for studying the SM sensitivity of ribosomes isolated from the SM-producing variant of *S. griseus*. At present we can use the ribosomes only of younger cells (about 16 hr), as the SM-phosphorylating activity can be washed out completely from the ribosomes of young cells, but not of older ones.

Materials and methods

Two *S. griseus* strains were used, a SM-producing (No. 52-1) and a nonproducing (No. 45-H) variant. The description of strains was published elsewhere [9]. Strains were cultivated in filtered soybean medium in a rotary shaker at 27 °C. Inoculation was carried out with a spore suspension to one million per ml of culture broth. Mycelia were harvested by centrifugation and the S-30 extracts (30 000 g supernatant) were prepared by the slightly modified

[10] method of LEGAULT-DEMARE and CHAMBLISS described for *Bacillus subtilis* [11]. The method eliminates the undesirable effect of proteolytic enzymes.

The S-30 extracts were dialysed overnight against the isolation buffer (0.01 M Tris-hydrochloride, 0.01 M $MgCl_2$, 0.06 M NH_4Cl , 5 mM Mg-Titriplex, 5 mM mercaptoethanol and 3.45 mM phenylmethanesulphonyl fluoride, with a final pH of 7.5), then S-100 fractions were prepared from the dialysed S-30 extracts by centrifugation at 110 000 g for 4 hr. Ribosomal pellets were washed once with the isolation buffer, then resuspended in S-100 fractions derived from the same and the other strain.

SM-phosphorylating capacity was assayed as described by OGAWA and PERLMAN [7]. The test system contained 0.5 ml quantities of 1.0 mM SM sulphate; 600 mM KCl; 100 mM magnesium acetate; 50 mM dithiothreitol; 40 mM ATP; 1.0 M potassium phosphate buffer pH 7.0; the cell-free extract and 1.5 ml of M/15 potassium phosphate buffer pH 7.0. Mixtures were incubated at 30 °C. Samples were taken at 0, 10 and 30 min, as longer incubations resulted in phosphorylation of the whole quantity of SM sulphate added. Samples were kept in boiling water for 3 min, then the residual SM sulphate was determined by bioassay using *B. subtilis* ATCC 6633 strain as the test organism. Results were expressed in μ g of inactivated SM per mg protein per hr.

SM could be regenerated by treatment with alkaline phosphatase at pH 8.5 at 37 °C for 12 hr.

The reaction mixture for *in vitro* polypeptide synthesis [12] contained per ml: HEPES (*N*'2-hydroxyphenylpiperazine-*N*'2-ethane-sulphonic acid) pH 7.8, 50.0 μ mol; KCl 50.0 μ mol; $MgCl_2$ 12.5 μ mol; dithiothreitol 12.5 μ mol; ATP 2.5 μ mol; GTP 0.1 μ mol; phosphocreatine 12.5 μ mol; creatine phosphokinase 70.0 μ g; 19 nonradioactive amino acids 25 nmol each; *E. coli* tRNA 10.0 A_{260} units; polyuridylic acid (poly-U) 625 μ g; S-30 fraction or ribosomes plus S-100 fraction, 25.0 A_{260} units; [^{14}C]phenylalanine ([^{14}C]Phe) 25 nmol (115 mCi per mmol). Incubation took place at 37 °C for 30 min. Samples were taken onto filter paper disks (Whatman 3 MM), which were then treated by the method of MANS and NOVELLI [13]. The radioactivity of [^{14}C]Phe incorporated into the hot trichloroacetic acid-insoluble polymer was measured in a Nuclear Chicago Isocap/300 liquid scintillation counter.

Protein was determined by the Folin method [14], with bovine serum albumin as standard.

Results

Figure 1 shows the SM-phosphorylating activity of cell-free extracts (10 000 g supernatants), SM-production, and the growth curve of *S. griseus* 52-1. SM-phosphorylating activity could be detected as early as 16 hr, when

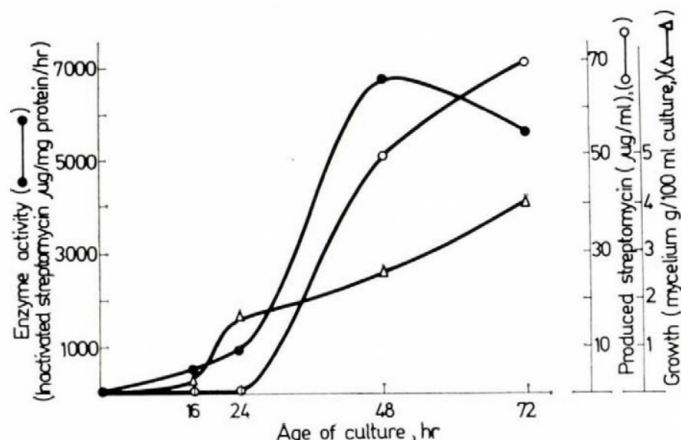


Fig. 1. SM-phosphorylating activity of cell-free extracts (10 000 g supernatants), SM-production and the growth curve of *S. griseus* No. 52-1. (Details in text)

the bioassay showed no SM in the culture broth. After 24 hr it increased rapidly, parallel to the SM-production.

At about 16 hr of age, the SM-phosphorylating activity was localized mainly to the S-100 fraction, and the washed ribosomes showed no SM-phosphorylating capacity. The cell-free extracts and subcellular fractions of the SM-nonproducing variant of *S. griseus* were inactive in SM-phosphorylation under the given circumstances.

The amino acid incorporating activity of cell extracts was examined in a poly-U acid directed [^{14}C]Phe-incorporating system. The S-30 fractions prepared from cultures of different stages of the life-cycle showed considerably different polyphenylalanine polymerizing capacity (Fig. 2). The highest activity

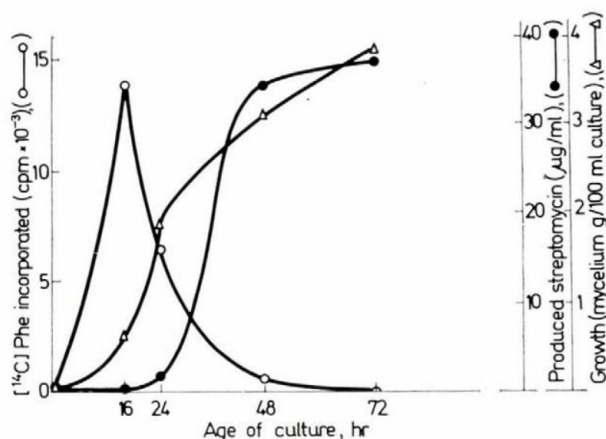


Fig. 2. [^{14}C]Phe-incorporating activity of S-30 fractions obtained from cultures in different stages of life-cycle, SM-production and growth of *S. griseus* No. 52-1

appeared at the age of 16–20 hr. The S-30 fractions obtained from the SM-nonproducing strain (45-H) showed the same property in the amino acid incorporating experiments (unpublished). This means that the change (decrease) of Phe-polymerizing capacity of S-30 fractions from the SM-producing strain may be related to the age of the cells and not to SM production. The phenomenon is under investigation.

For studies of SM-sensitivity of ribosomes from the SM-producing strain we had to select the 16 hr age, as the SM-inactivating capacity could be washed out perfectly from ribosomes of mycelia harvested at this age.

Table I summarizes the results concerning the effect of SM on the incorporation of Phe by the S-30 fractions from both strains of *S. griseus* and *E. coli* K-12, and by washed ribosomes of the SM-producing strain plus the S-100 fraction from the nonproducing variant. As expected, the inhibitory effect of SM on Phe incorporation by the S-30 fractions of strain 52-1 depended on the age of the culture from which the S-30 had been isolated, as the SM-

Table I

*Effect of streptomycin on the [¹⁴C]Phe incorporating activity of cell-free extracts isolated from streptomycin-producing (52-1), the nonproducing (45-H) *S. griseus* strains and from *E. coli* K-12*

Strain	Age of culture, hr	Cell-free extract	Inhibition by 30 µg/ml streptomycin, per cent
52-1	16	S-30	40
52-1	24	S-30	11
52-1	48	S-30	0
45-H	16	S-30	72.8
45-H	24	S-30	78.5
<i>E. coli</i> K-12		S-30	79
52-1	16	ribosomal fraction plus S-100 fraction	81.5
45-H	16		

Results are expressed by the average of three unrelated experiments

inactivating capacity of cell extracts increased with the age of the mycelia (see Fig. 1).

The S-30 extracts derived from the nonproducing *S. griseus* strain showed the same sensitivity to SM as did the S-30 fraction of *E. coli* K-12, because these strains had no SM-phosphorylating enzymes. The amino acid incorporating activity of the mixture, containing washed ribosomes from the SM-producing strain and S-100 fraction from the nonproducing variant of *S. griseus*, was inhibited with 30 µg/ml SM in the same degree as the activity of S-30 fraction from *E. coli* K-12 or from the nonproducing strain, demonstrating that if the SM added to the amino acid polymerizing system was not converted into an inactive form, could bind to the ribosomes of the SM-producing strain and inhibit their function. The S-100 fraction from the nonproducing strain had no amino acid incorporating activity in the poly-U system, showing that it did not contain ribosomes.

These results offer direct evidence that ribosomes of the 16 hr SM-producing *S. griseus* strain are as sensitive to SM as are the ribosomes of the non-producing strain or of *E. coli* K-12.

Discussion

The SM-sensitivity of ribosomes derived from a SM-producing strain was examined. There were some difficulties we had to overcome in order to get reproducible results. One of them was the perfect elimination of SM-phosphorylating activity from ribosomes of the SM-producing strain. It could be

carried out only with ribosomes of young (about 16 hr) cultures, though washing of ribosomes isolated from older (24–48 hr) cells, with buffer of high ionic strength has not yet been tried.

Another problem was how to isolate active amino acid polymerizing cell-free extracts from the SM-producing *S. griseus*. We namely found that S-30 fractions, active in poly-U acid directed polyphenylalanine synthesis, could be obtained from the SM-nonproducing *S. griseus* cells, if the proteolytic enzymes were inhibited during the isolation procedure [11]. We obtained the same results with the SM-producing strain, i.e. cell-free extracts (S-30 fractions) isolated without protease inhibitors by the classical method of NIRENBERG and MATTHAEI [12] were inactive in the poly-U system (unpublished results). Similar data were reported for another SM-producing *S. griseus* by CELLA and VINING [15]. In their opinion there was an unknown factor in the cell extract, which inhibited its amino acid incorporating activity in the poly-U system. Thus, by the inhibition of proteolytic enzymes we succeeded in isolating a well functioning protein synthetizing machinery also from the SM-producing strain.

The results presented indicate that the ribosomes of a SM-producing *S. griseus* strain, at least at the age of 16 hr, have the same sensitivity to SM as have the ribosomes of the nonproducing strain or of *E. coli* K-12.

Though the SM-sensitivity of ribosomes from *S. griseus* cells, which are actually producing SM, is not known, it seems that the compartmentalization of cells, the SM-inactivating mechanism and the decreased permeability to SM during SM production [15] are sufficient to protect the organism from its own product.

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INDUCTION OF INTERFERON AND RADIOPROTECTIVE ACTIVITY OF POLYRIBOGUANYLIC-POLYRIBOCYTIDYLIC ACID COMPLEX IN MICE

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The effect of polyriboguanylic-polyribocytidylic acid complex was investigated against acute X- and prolonged ^{60}Co -gamma irradiation. Prophylactic administration of the polyribonucleotide complex increased endogenous spleen colony formation and the percentage of survival of irradiated BALB/c mice. The most pronounced effect was observed when the animals had been irradiated at the time of maximum interferon accumulation in blood, i.e. 24 hr after interferon induction by the polyribonucleotide complex.

Radioprotective activity of interferon inducers such as Newcastle Disease Virus, *Escherichia coli* endotoxin, polyriboinosinic-polyribocytidylic acid (poly rI : rC), tilorone and Acranil has been reported previously. The interferon inducers produced (i) a 2.0–2.5fold increase of the number of nucleated bone marrow cells, and (ii) a 2–4fold decrease of aberrant mitosis in proton-irradiated mice [1, 2]. An increase of the number of endogenous colonies in spleen as well as the percentage of surviving X- and ^{60}Co -gamma irradiated mice after administration of the interferon inducers was also shown [3, 4].

Antiviral activity and interferon inducing capacity of polyriboguanylic-polyribocytidylic acid complex (poly rG : rC) has convincingly been demonstrated [5, 6]. Here we report the radioprotective effect of poly rG : rC against acute X- and prolonged ^{60}Co -gamma irradiation.

Materials and methods

Mice. Inbred BALB/c mice of both sexes weighing 26–28 g (12–14 weeks old) were used.

Interferon inducers. Polyriboguanylic-polyribocytidylic acid complex (poly rG : rC) was kindly supplied by the Institute of Macromolecular Compounds (Academy of Sciences, Leningrad, USSR). Polyriboinosinic-polyribocytidylic acid complex (poly rI : rC) was obtained from Calbiochem. Both compounds were administered intraperitoneally (i.p.) in doses of 8 mg/kg. Poly rG : rC was injected at different intervals before or after irradiation.

Total body irradiation. For total body irradiation fifteen mice were put in separated plexiglass holders and irradiated with X-rays, using a THX apparatus (200 kV, 20 mA, 0.5 mm Cu filter); exposure rate 44 R per min, dose 450 or 760 R [7]. In the prolonged experiments mice were irradiated with ^{60}Co -gamma rays (exposure rate 0.7 rad per min; total dose, 700 rad).

Endogenous spleen colony formation test. On the 10th day after irradiation the mice were killed by cervical dislocation; their spleens were dissected free of adherent tissues, fixed in Bouin's fixative and the macroscopic colonies were counted [8].

Survival of mice. Percentage of surviving mice was recorded on the 30th day after ^{60}Co -gamma irradiation.

Interferon assay. Interferon was assayed by the cytopathic effect reduction method on monolayer L cell cultures using encephalomyocarditis virus as a challenge [7].

Results and discussion

Table I shows interferon induction in mice by poly rG : rC and poly rI : rC. In contrast to poly rI : rC, poly rG : rC induces a delayed production of circulating interferon in sera of mice with a maximum level 24 hr after administration. This observation supports the results reported by VILNER *et al.* [9].

Table I

Interferon induction in mice by poly rG : rC

Treatment of mice (8 mg/kg i.p.)	Interferon titer in 0.5 ml serum* after			
	2	6	24	48
	hours			
—	< 5	< 5	< 5	< 5
poly rG : rC	< 5	< 5	160	10
poly rI : rC	320	80	< 5	< 5

* 10 mice per group

The effect of poly rG : rC administration on endogenous colony formation in the spleen of mice irradiated with X-rays (450 R) is shown in Table II. Intraperitoneal injection of poly rG : rC (8 mg/kg) resulted in an increase of the number of colonies if the drug was given 24–48 hr before irradiation. The

Table II

Endogenous spleen colony formation in BALB/c mice treated with a single dose of poly rG : rC before or after acute X-irradiation (450R)

Time of treatment with poly rG : rC (8 mg/kg i.p.)	Mean colony count ($\pm$ s.d.) (10–15 mice per group)
—	1.1 $\pm$ 0.3
2 hr before irradiation	1.2 $\pm$ 0.6
6 hr before irradiation	1.6 $\pm$ 0.5
24 hr before irradiation	18.7 $\pm$ 0.8
48 hr before irradiation	7.4 $\pm$ 0.9
1 hr after irradiation	2.1 $\pm$ 0.9

most pronounced increase was observed when the mice had been irradiated at the time of maximum interferon accumulation in blood, i.e. 24 hr after poly rG : rC administration.

Prophylactic treatment with poly rG : rC resulted in an increase from 5 to 40% in the survival of X-irradiated mice (Table III).

Administration of poly rG : rC 24 hr before irradiation was also effective against prolonged ^{60}Co -gamma irradiation, increasing the number of endogenous spleen colonies (Table III).

Table III

Effect of prophylactic treatment of BALB/c mice with poly rG : rC on survival after acute X-irradiation and on endogenous spleen colony formation after prolonged ^{60}Co -gamma irradiation

Treatment 24 hr before irradiation	Per cent of surviving mice 30 days after X-irradiation (700 R)	Mean colony count ($\pm$ s.d.) 10 days after ^{60}Co -gamma irradiation (0.7 rad/min, total dose: 700 rad)
—	5 ^a	1.5 $\pm$ 0.7 ^b
poly rG : rC (8 mg/kg i.p.)	40	28.0 $\pm$ 1.8

^a 20 mice per group

^b 15 mice per group

The results indicate a certain role of interferon in the radioprotective activity of poly rG : rC. Prophylactic administration of homologous interferon preparations is known to increase the number of endogenous colonies in the spleen of mice [10] and the survival of mice after irradiation [11].

One of the possible mechanisms of the radioprotective activity of interferon inducers can be a transient interruption of the cell cycle by a newly synthesized interferon at the phases G and S [12, 13] which are less sensitive to irradiation [14].

The interferon inducing capacity of thiol compounds reported recently [15, 16] may also indicate the role of interferon in the radioprotective effect of some interferon inducers and radioprotectors [4].

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SHIGELLA DYSENTERIAE 1-LIKE CYTOTOXIC ENTEROTOXINS PRODUCED BY SALMONELLA STRAINS*

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A *Salmonella enteritidis* strain produced a cytotoxin in addition to heat-labile (LT) and heat-stable (ST) enterotoxins. Two strains of serotypes *Salmonella kapemba* and *Salmonella thompson* were LT and ST negative, but exhibited a cytotoxic effect. After Sephadex G-100 fractionation of the crude *S. enteritidis* material, some high and low molecular fractions had both cytotoxic and cytotoxic activities. Of the two other salmonellae, only some high molecular fractions contained the cytotoxic substance. Neutralization experiments revealed an antigenic relationship between the cytotoxins studied and *Shigella dysenteriae* 1 enterotoxin. On the basis of cross neutralization and other data, it seems that cytotoxic and LT-like characters are carried by the same molecule. In *S. thompson* and *S. kapemba* the LT fails to exert a biological effect, although it is antigenically related to the LT of *Escherichia coli*.

In recent years, besides the “classical” enterotoxin types such as the LT, ST and *Shigella dysenteriae* 1 enterotoxins of *Enterobacteriaceae* some other, somewhat “intermediate” types were also described. A special ST produced by *Shigella flexneri* strains was observed by us [1, 2]; it is heat and acid stable, active only in the ST specific suckling mouse test, and with its high molecular weight and antigenicity shows a close resemblance to *Escherichia coli* LT. O'BRIEN *et al.* [3] found a strain of *S. flexneri* type 2a producing *S. dysenteriae* 1-type enterotoxin. We demonstrated [4] that strain (M42-43), too, had a *S. flexneri*-like high molecular ST activity and assumed that the same molecule carried both toxic activities.

In the present paper, similar “intermediate, hybrid-like” enterotoxins of *Salmonella* strains will be described.

Materials and methods

Strains. *S. enteritidis* No. 724, *S. kapemba* No. 5, and *S. thompson* No. 14 were isolated in the routine laboratory of our institute. *S. dysenteriae* 1 No. 16 and *E. coli* No. 263 O8: K87, K88a, b; H19 (LT+ST-) strains of our collection served as controls.

Sera. Antitoxic sera were prepared in rabbits by subcutaneous inoculations of Sephadex G-100 active fractions mixed with complete Freund adjuvant (Difco) according to CALLAHAN *et al.* [5]. After bleeding, the sera were collected, sterilized by filtration, distributed in vials, and stored at -20 °C without chemical preservation.

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Media and cultivation. For enterotoxin production the strains were cultured in a modified [1, 2, 4, 6] Sakazaki medium in shaker at 37 °C for 18 hr. In the case of *S. dysenteriae* 1, cultivation was prolonged for 36 to 48 hr.

Crude enterotoxin preparations. Ultrasonic lysis, alkaline extraction, concentration by freeze-drying, as well as Sephadex G-100 fractionation, have been described previously [1, 2, 4].

Enterotoxin assay has also been described previously [1, 2, 4, 6].

Neutralization experiments. In AV-3 (a cell line of human amniotic origin) the antibodies to cytotoxicity, while in CHO (Chinese Hamster Ovary cell line) cells the antibodies to cytotoxicity were tested. The technical data were published earlier [2, 4]. Microplates (Linbro TC-IS-EB-96) were used for the assays. Neutralization values for the antisera were calculated taking into account the dilutions and volumes used.

Results

1. *Enterotoxigenicity of crude lysates and extracts.* *Salmonella* strains isolated by the routine laboratory of the institute were investigated for cytotoxicity and cytotoxicity. In the first phase the ultrasonic lysates were screened in AV-3 and CHO cells. Three of the strains tested were found to be cytotoxic: one *S. enteritidis* (No. 724), one *S. kapemba* (No. 5) and one *S. thompson* (No. 14) strain. The *S. enteritidis* lysate was active also in CHO culture while the other two only in AV-3. Preliminary neutralization of cytotoxicity by *S. dysenteriae* 1 antitoxic serum was attempted; the positive result indicated the "shigella-like" nature of the cytotoxin(s).

In the second phase of our experiments the lysates were tested in other enterotoxin assay systems. Ligated rabbit loop test, blueing test according to EVANS *et al.* [7], and the mouse pad oedema test were performed for the LT, while for the ST the suckling mouse test was used. Preliminary tests in CHO and AV-3 cells were repeated.

Data obtained are summarized in Table I. It shows that the lysate of *S. enteritidis* was positive in all the enterotoxigenicity tests, indicating that the lysate contained both LT and ST. The manifestation of LT activity without purification (being not masked) was somewhat surprising [8]. With the two lysates derived from other salmonellae neither ST nor LT, but a cytotoxic effect was observed. In view of these findings the two lysates were concentrated to 1 : 10 by freeze-drying and certain tests were carried out, but no activities could be registered. Similarly, the extracts prepared by the method of VAN HEYNINGEN and GLADSTONE [9] applied originally to obtain *S. dysenteriae* 1 toxin, were negative for LT and ST and their cytotoxic effect was not superior to that of the ultrasonic lysates.

2. *Experiments with Sephadex G-100 fractions.* Two hundred mg of each freeze-dried and dissolved ultrasonic lysate were filtered through the same Sephadex G-100 column. Fifty fractions of 5 ml each were collected and stored at -20 °C. The LT active material of *E. coli* No. 263 and the cytotoxic material of *S. dysenteriae* 1 No. 16, obtained earlier in a similar way served for comparison of the molecular weight ranges. Each fraction was tested for cyto-

Table I

Different activities of cell-free materials of salmonellae tested for enterotoxigenicity

Activities	Limit of positivity	<i>S. enteritidis</i> No. 724 ultrasonic lysate	<i>S. kapemba</i> No. 5 ultrasonic lysate	<i>S. thompson</i> No. 14 Heyningen extract
Ligated rabbit loop test (Index values)	1.0	1.2	0.1	0.1
"Delayed PF" test on rabbit skin (blueing area/mm ²)	78.5	176.0	< 3.14	< 3.14
Mouse foot oedema test (1 biol. Unit = 0.75 mg of oedema)	1	1.1	0.54*	0.69*
Cytotonic effect in CHO cells (titre; without limit)	—	1 : 4	< 1 : 4*	< 1 : 4*
Suckling mice dilatation test (bowel/body w. index)	0.07	0.098	0.065*	0.066*
Cytotoxic effect in AV-3 cells (titre; without limit)	—	1 : 4	1 : 20	1 : 20

* Experiments repeated with materials concentrated about 1 : 10 by freeze-drying

toxicity in AV-3 cells and for LT activity in CHO cells. Results are summarized in Table II.

In the case of *S. enteritidis* two active fraction groups were found. One in the higher molecular weight range showed a rather narrow band of 60 to 70 ml, while the other in the low molecular weight range gave a wider band of 160 to 200 ml. Both high and low molecular weight bands had cytotoxic activity for AV-3 cells as well as cytotonic effect in CHO cells. Fractions of the

Table II

Cytotoxic and cytotonic activity of Sephadex G-100 fractions concentrated by freezing and drying of crude ultrasonic salmonella lysates

Fraction, volume, ml	Reciprocal titre values of active fractions					
	<i>S. enteritidis</i> No. 724 in		<i>S. kapemba</i> No. 5 in		<i>S. thompson</i> No. 14 in	
	AV-3	CHO	AV-3	CHO	AV-3	CHO
0- 60	—*	—	—	—	—	—
60- 70	16	20	16	—	16	—
70-160	—	—	—	—	—	—
160-200	20	20	—	—	—	—

* = < 1 : 4

two other salmonellae, in agreement with previous results, had only cytotoxic activity occurring in the high (60 to 70 ml) molecular weight fractions.

3. *Neutralization experiments.* The effect of antitoxic immune sera on cytotoxicity of our substances was tested in AV-3 cells. Antiserum to *S. dysenteriae* 1 toxin, antitoxic serum produced against the two salmonella materials showing cytotoxicity only, and an *E. coli* (No. 263) anti-LT serum were tested. Results are summarized in Table III.

On the basis of preliminary experiments the neutralizing effect of anti-*S. dysenteriae* 1 toxin was expected, but it neutralized the salmonella cytotoxins in a considerably lower degree than did the homologous toxin. This may indicate that the salmonella and *S. dysenteriae* 1 cytotoxic enterotoxins are not identical though antigenically related. The neutralizing activity of the anti-LT serum against the CHO active fractions of *S. enteritidis* was also expected, but not against those of the other salmonellae. Though these substances had no LT activity, the anti-LT serum neutralized them at the same rate as did the *S. enteritidis* toxin (Table III). In reciprocal experiments an

Table III

Neutralization of salmonella enterotoxins in AV-3 cell culture by antitoxic sera

Antitoxic sera	<i>S. enteritidis</i> No. 724		<i>S. kapemba</i> No. 5	<i>S. thompson</i> No. 14	<i>S. dysenteriae</i> 1 No. 16
	HMF _r	LMF _r	HMF _r	HMF _r	HMF _r
	2 TCD ₁₀₀				
<i>S. dysenteriae</i> 1	243*	322	500	243	5263
anti- <i>E. coli</i> LT	666	322	285	243	< 80
anti-salmonella**	.	1000	.	500	< 80

HMF_r = high molecular weight fraction (about 100 000 dalton), LMF_r = low molecular weight fraction (about 5–10 000 dalton) estimated by Sephadex G-100 fractionation

TCD₁₀₀ = tissue culture cytotoxic dose of 100% effectivity

* Neutralization capacity of sera expressed per ml, taking into account the effective dilution and the volume

** Prepared by mixing of 60 to 70 ml Sephadex fractions of *S. kapemba* and *S. thompson*

antiserum prepared with the cytotoxic fractions of *S. kapemba* and *S. thompson* was used; it had a high homologous neutralizing capacity, but did not neutralize the partially purified *S. dysenteriae* 1 toxin. This may support the assumption that the salmonella cytotoxins are related only to *S. dysenteriae* 1 enterotoxin (Table III).

The next series of experiments was aimed at clarifying the relationship between salmonella enterotoxins and heat labile *E. coli* enterotoxin. The neutralizations were carried out in CHO cells (Table IV). Due to the low cyto-

Table IV
Neutralization of E. coli LT in CHO cells
by antitoxic sera

Antitoxic sera	<i>E. coli</i> O8:K87, K88a, b:H19 LT+ (No. 263) Sephadex G-100 high molecular weight fraction — 2 TCD ₃₀
anti-salmonella	243*
anti- <i>E. coli</i> 263 LT	666
anti- <i>S. dysenteriae</i> 1	< 80

TCD₃₀ = tissue culture cytotoxic dose causing clearly observable cell elongation of about 30%.

* Neutralization capacity of sera expressed per ml, taking into account the effective dilution and the volume

tonic titres of *S. enteritidis* material, it was impossible to obtain reliable results and the neutralizing capacity of the salmonella antiserum and control sera was measured against *E. coli* LT. The salmonella antiserum of non-LT active salmonellae had fully neutralized the LT. The homologous anti-LT serum had somewhat higher value, while the heterologous *S. dysenteriae* 1 antitoxic serum was ineffective, as expected.

Discussion

KOUPAL and DEIBEL [10] and SANDEFUR and PETERSON [8] showed that salmonellae can produce ST and LT type enterotoxins. The latter could be neutralized by cholera antitoxin [11]. In our experiments a *S. enteritidis* strain produced similar enterotoxic activities. Nevertheless we found certain differences as compared to the data of SANDEFUR and PETERSON [8]. First, there was the "unmasked" character of LT in the crude preparation. It was more surprising to find cytotoxic activity in the crude as well as in the Sephadex filtered material. The preliminary neutralization test with anti-*S. dysenteriae* 1 serum revealed the relationship of this cytotoxin to the *S. dysenteriae* 1 toxin.

Studying the Sephadex G-100 fractions, two active peaks were found; they had both cytotoxic and cytotoxic activities. In the case of *S. dysenteriae* 1, a similar second, cytotoxic peak was described by KEUSCH and JACEWICZ [13]. The LT activity of such a low molecular weight fraction may suggest that both activities are carried by the same molecule. The two other salmonellae seem to produce only one *S. dysenteriae* 1-like enterotoxin without LT and ST activity.

Results of the neutralization experiments showed that in the case of *S. enteritidis* both active fractions can be neutralized by anti-*S. dysenteriae* 1 serum and by an anti-*coli* LT antiserum. The ten times lower effectiveness of

the anti-*S. dysenteriae* 1 serum against *S. enteritidis* cytotoxin as compared to the homologous toxin suggests an antigenic relationship rather than an identity. The fact that, demonstrating an unilateral relationship, *S. dysenteriae* 1 toxin cannot be neutralized by salmonella antitoxin, supports this suggestion. The finding that the low molecular fragment has both cytotoxicity and cytotonicity and can be neutralized by anti-*S. dysenteriae* 1 and anti-LT antisera, unequivocally suggests the existence of a single enterotoxin molecule carrying both the biological and the antigenic properties. This is in analogy to the *S. flexneri* strain M42-43 which produces a toxin with activities of *S. dysenteriae* 1 and also the special *S. flexneri* heat stable toxins [4].

The two other toxins produced by *S. kapemba* and *S. thompson* strains are somewhat different. These toxins showed only a cytotoxic character which could be neutralized not only by *S. dysenteriae* 1 antitoxin but also by anti-LT serum. The antiserum produced against their mixed active fractions had an anti-LT neutralizing capacity as shown in the reciprocal experiments. This may mean that this "class" of enterotoxins carry the partial antigenicity and immunogenicity, but not the activity of the LT.

Enterotoxin research has revealed three "classical" types produced by *Enterobacteriaceae*: the choleraen-like LT, the heat stable (ST) and the cytotoxic enterotoxin of *S. dysenteriae* 1. LT and ST seem to be widespread and identical in the *Enterobacteriaceae*, although KLIPSTEIN and ENGERT [14] found that the LT and ST produced by *E. cloacae* differed in antigenicity from those produced by *E. coli* or *K. pneumoniae*. The toxic properties of *S. flexneri* strain M42-43 isolated by O'BRIEN *et al.* [3] has already been mentioned. Our findings in connection with salmonella enterotoxins support the existence of enterotoxins other than the "classical" types.

It is almost impossible to compare precisely an isolated toxin (LT, ST or cytotoxin) with the classical types. KONOWALCHUK *et al.* [15], however, described comparative results testing a number of *E. coli* filtrates in Vero, CHO and Y-1 cell lines and they could observe cytotoxicity caused by numerous filtrates in Vero cells. *S. dysenteriae* 1 antitoxins were found by KEUSCH and JACEWICZ [16] in sera of patients convalescing after *S. flexneri* or *S. sonnei* dysentery. These data may suggest that the occurrence of strains producing "non-classical" types of enterotoxins is not exceptional.

It is common to all enterotoxins that they provoke fluid and electrolyte secretion through the cAMP mediator system. On the other hand, they show wide varieties in molecular weight, biological activity and antigenic structure. Theoretically, the background of these divergencies can be attributed to a single gene which had undergone mutational changes and perhaps hybridization processes. The *tox* gene in *V. cholerae* (at least in case of 569B strain) is well known to be of chromosomal origin [17]. Studies concerning the genetical background of LT or ST produced by *E. coli* revealed [18, 19] that the *tox*

gene was carried by a plasmic element (Ent). In a recent paper TAKEDA and MURPHY [20] have shown that the information of a mitomycin C inducible LT is of phage genome origin, and that the *tox* gene might be a translocable element. In this case the widespread nature of enterotoxigenesis is only natural. On the other hand, information carriers such as phages or plasmids, may easily be involved in hybridization processes. The toxins of M42-43 and salmonellae described in this paper, can be suggestive examples of such speculations.

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ISOLATION OF CYTOPATHOGENIC ROTAVIRUS FROM NEONATAL CALVES

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Rotavirus was isolated in rolled calf kidney cultures from the intestinal contents of a calf suffering from diarrhoea. The cytopathic effect was demonstrated in native cultures as well as in stained preparations. The affected cells were elongated, became sickle-like in shape, disintegrated and detached from the monolayer. Virus-specific direct immunofluorescence ran parallel, in both time and intensity, with the cytopathic effects. Immuno-electron microscopy showed aggregated virus particles corresponding in size, shape and structure to *Rotavirus* virion.

In 1969, MEBUS *et al.* [1] isolated in Nebraska (USA) a previously unknown virus from faecal samples of neonatal calves suffering from diarrhoea and proved the aetiological role of the virus. The disease was described as Nebraska calf diarrhoea and the virus was designated as a reo-like virus.

Since then, the presence of the virus has been proved in Canada [2], Australia [3], England [4], Northern Ireland [5] and, more recently, in France. Similar reovirus-like viruses have been found in diarrhoeic faecal samples from children [6–9], pigs [10, 11], lambs [12], suckling mice [13], etc. The viruses isolated from different species are antigenically closely related as it has been demonstrated by complement fixation, agar gel diffusion, immunofluorescence (IF) and immuno-electron microscopy (IEM) [7, 14]; they can be distinguished from one another by virus neutralization test [14, 15]. All these viruses are now classified in the *Rotavirus* genus of the *Reoviridae* family [16].

Up to now, isolation in tissue cultures of four cytopathogenic rotavirus strains has only been reported [17–19]. In Hungary, we [20] proved the presence and the aetiological role of rotavirus in severe epizootic diarrhoea by experimental infection of animals, electron microscopic examination of the intestinal contents of naturally and experimentally infected animals, and by IF of intestinal epithelial cells. In the present paper, isolation and characterization of a bovine rotavirus isolate is reported.

Materials and methods

Virus isolation. Faecal samples from calves with diarrhoea as well as small intestinal segments and mesenteric lymph nodes from an exsanguinated calf were examined. Intestinal segments, together with the thin contents, were homogenized and sonicated, then diluted in 4 volumes of Hanks's balanced solution and centrifuged at 4000 rpm at 4 °C twice for 20 min.

Fetal calf kidney and calf testicle cell cultures were prepared as usual and the cultures were allowed to adsorb virus from the inoculum for 90 min. The inoculated cultures were incubated at 37 °C, some in a rolling drum (12 rev/hr), others as stationary cultures. Cultures were examined in the native state or fixed in methanol and stained with haematoxylin-eosin.

Immuno-electron microscopy (IEM). The anti-rotavirus serum (H48) used in these tests had been raised in gnotobiotic calves in the Institute for Research on Animal Diseases (Campton, England) and the fluid phase of cytopathic cultures, clarified by centrifugation and passed through a filter 0.45 µm in diameter, was used as virus. Four volumes of serum dilution from a twofold dilution series from 1 : 10 to 1 : 60, was added to one volume of virus. The mixtures were kept at room temperature for 1 hr, then at 4 °C overnight. This was followed by centrifugation at 31 000 g for 10 min in a Janetzki vac 60 centrifuge. The pellet was re-suspended in 0.1 ml distilled water. Negatively stained preparations were examined in a Tesla electron microscope type BS 613.

Immunofluorescence studies. Cell cultures showing CPE were examined by a direct IF method with FITC-conjugated anti-rotavirus sera. One of the conjugates had been kindly supplied by Dr. C. A. MEBUS (Nebraska, USA), our own conjugate was prepared from a rabbit antiserum raised by us against the bovine *Rotavirus* reference strain NCD [21, 22]. The preparations were examined under a Zeiss microscope NU-2.

Results

Isolation of the H. Cs. strain. From the third day after inoculation with intestinal contents, elongation of certain cells was observed in rolled calf kidney cultures. Shrinkage of the elongated cells made them sickle-like and refractive, and they gradually separated from the mass of the monolayer. Some of them appeared for a time attached to the monolayer by one pole while the cell itself was floating in the maintenance fluid. Sometimes, several

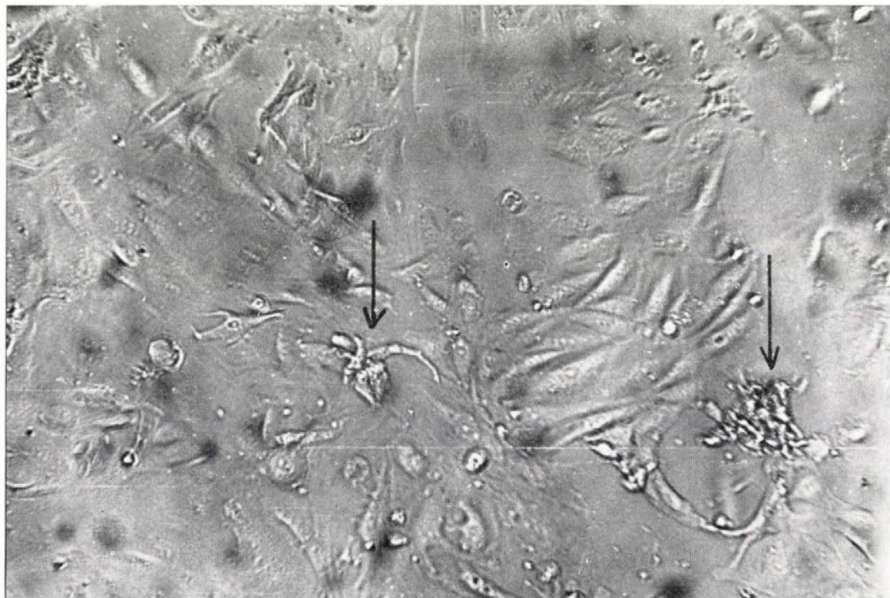


Fig. 1. Cytopathic changes in native calf kidney cell cultures infected with the H. Cs. strain of bovine rotavirus. $\times 200$

adjacent cells formed a brush-like structure (Fig. 1) until detached from the culture. The process was the most pronounced on the 4th or 5th day. On the 7th or 8th day, cells floating in the medium were the only sign reminding of the earlier CPE.

In passages 2 to 4, affected cells were decreasing in number, but after the 6th passage CPE suddenly became more pronounced. In further passages, the first signs of CPE appeared 48 to 72 hr after inoculation. By the 10th passage the virus titre had reached 10^4 TCID₅₀/0.2 ml.

In haematoxylin-eosin preparations, the affected cells had a dark shrunken cytoplasm and disintegrated nuclei (Fig. 2).

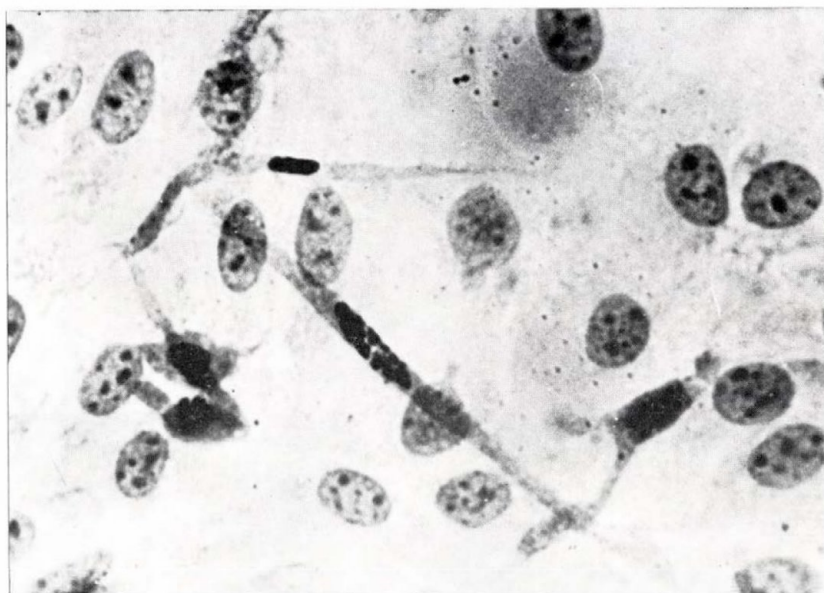


Fig. 2. Cytopathic changes in stained calf kidney cell cultures infected with the H. Cs. strain of bovine rotavirus. $\times 400$

We failed to observe any CPE in stationary cultures of calf kidney cells. After 8 serial passages in rolled cultures weak CPE similar to that described above was observed appearing as late as on the 7th or 8th day.

Immune-electron microscopy. In negative contrast preparations, large aggregates containing great numbers of virus particles agglutinated by the antiserum were seen. The particles, 65 to 70 nm in diameter, were morphologically characteristic of the *Rotavirus* virion, especially if the serum dilution was 1 : 40 (Fig. 3).

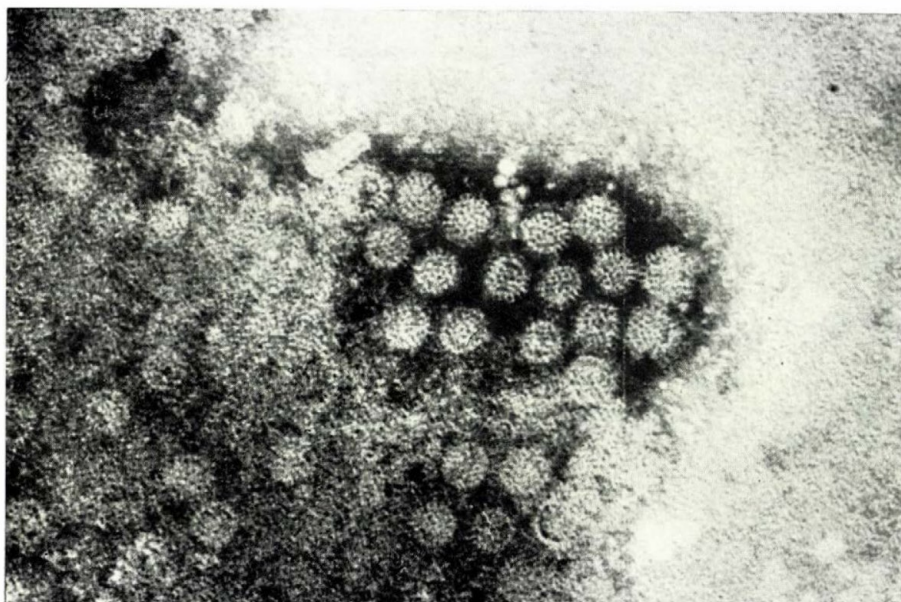


Fig. 3. Typical rotavirus particles (H. Cs. isolate) agglutinated by 1 : 40-diluted antiserum $\times 108\ 000$



Fig. 4. Granular cytoplasmic fluorescence in a calf kidney cell culture 30 hr after infection with the H. Cs. isolate

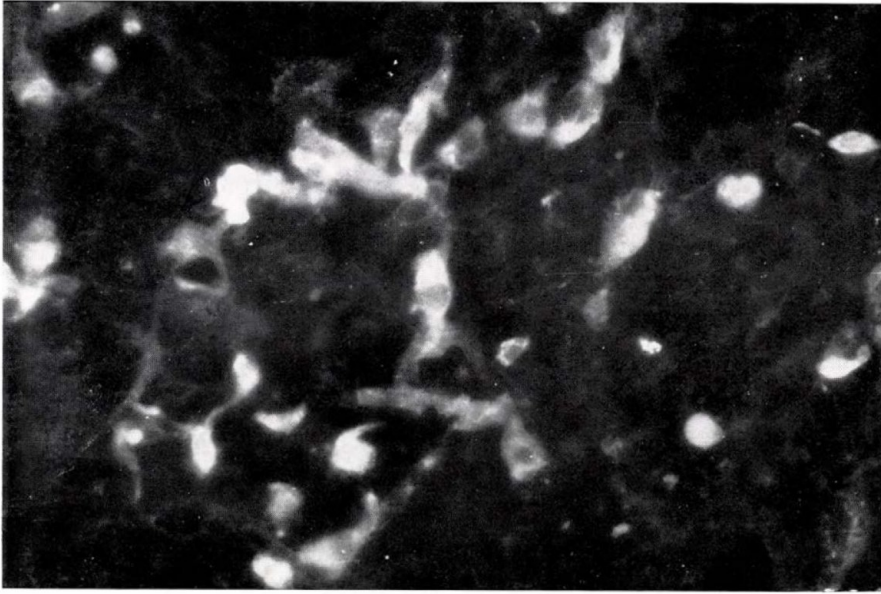


Fig. 5. Diffuse cytoplasmic fluorescence 72 hr after inoculation with the H. Cs. strain

Immunofluorescence studies. Intensive focal IF had appeared in the cytoplasm by the 30th hr after virus infection (Fig. 4). By the 72nd hr, the fluorescence became diffuse in the whole cytoplasm (Fig. 5). Mainly the cells showing CPE in native preparations were brightly fluorescing. In passages 2 to 4, fluorescent cells diminished in number, but the fluorescence did not change in intensity. From passage 7 or 8 on, parallel with the growing number of cytopathic cells, fluorescence increased again.

Pathogenicity of the isolate. Colostrum-free calves were infected with a low-passage material or with the 12th passage material of the isolate. The inocula were pretreated with chloroform twice. The characteristic symptoms appeared after a latency of 24 hr, regardless of the passage number.

Discussion

Up to now, 4 cytopathogenic bovine rotavirus strains have been isolated in tissue culture [17-19]. MEBUS *et al.* [17] as well as McNULTY *et al.* [5] isolated strains from faecal samples. Our attempts at isolating the virus from faecal samples have failed although disintegrated virus particles were demonstrated in the inocula by electron microscopy. On the other hand, we succeeded in isolating virus from the intestinal contents of a calf that had been exsanguinated in the earliest period of the disease.

McNULTY *et al.* [19] pointed to the advantages of rolling cultures in isolating rotavirus. The CPE was considerably more pronounced in rolling cultures than in stationary ones, but the virus titre was not influenced by rolling. We isolated the H. Cs. strain in rolling cultures while we failed to isolate virus from the same sample in stationary cultures. Successful infection of stationary cultures needed 10 consecutive passages in rolling cultures and undiluted inoculum.

The CPE observed by us in native and stained cultures was characteristic and did not change in character during serial passages. The lesions were similar to those reported by WELCH and TWIEHAUS [23] and McNULTY *et al.* [19], except that we never found vacuolation and cytoplasmic inclusions.

The H. Cs. strain retained its virulence for calves through 12 passages in cell cultures.

We found bright cytoplasmic IF, first focal then diffuse in character, running parallel to the CPE. Cultures showing no CPE were not fluorescing. MEBUS *et al.* [1, 17] working with the NCD strain, often found IF in cell cultures which had been inoculated with virus dilutions causing no CPE.

According to IEM and IF studies, the H. Cs. strain is antigenically closely related to, or identical with, the bovine *Rotavirus* reference strain NCD.

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ANTITOXIC IMMUNITY AGAINST THE SO-CALLED LUNG TOXIN PRODUCED BY *ESCHERICHIA COLI**

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Cross neutralization test with antisera to crude haemolysins produced by some *Escherichia coli* strains indicated that there were no antigenic differences among the haemolysins tested. These crude preparates showed definite cytotoxicity which could also be cross neutralized by "antihaemolysin" sera. Neutralization experiments were performed in mouse lung test with homologous and heterologous anti-haemolysin sera, and with O and OK sera to the wild type strain and its toxic R mutant. The results showed that the immunity in the mouse lung model is antitoxic and antibacterial.

In a previous paper [1] it was shown that some strains of *Escherichia coli* produced a toxic substance. This causes in mice haemorrhagic, fatal lung oedema, it is necrotoxic in the rabbit skin and mouse foot pad tests. The material is cytotoxic and can easily be assayed in AV-3 cell culture. In addition there are data suggesting its identity with an alpha-type haemolysin.

In this paper we present further data on the antigenicity of the supposed toxic haemolysin and the identity of the "lung toxin", "cytotoxin" and haemolysin. Experiments carried out in the mouse lung model were aimed to elucidate the role of antitoxic and antibacterial immunity.

Materials and methods

Strains. The lung and cytotoxic and haemolytic *E. coli* O4 : K12(L) : H5 strain designated "Kreka" was used, as in earlier studies, as well as the strain 281/54 (O4) with similar character, though the haemolysin proved to be directed by a Hly plasmid. A further, so-called "oedema disease strain" designated M-S-15 (O139) was kindly sent by Dr. B. NAGY (Veterinary Health Institute, Szombathely, Hungary).

Hly- EMS-induced mutants were isolated from the strain Kreka. R mutants were also isolated.

Media and cultivation. For the production of alpha-haemolysin the methods described by SMITH [2] were followed. A high inoculation dose (about 5×10^7 germs per ml) of overnight broth culture was transferred into fresh broth to which pieces of meat had been added before filtration and second autoclaving. After 2 hr incubation at 37 °C, the cultures were centrifuged sharply and the crude supernatants sterilized by membrane filtration were tested for haemolytic activity.

Ultrasonic lysates were prepared from shaken cultures in a modified Sakazaki medium [3], in which the Myosat was replaced with Levinthal broth [4]. After incubation at 37 °C for 18 hr, bacteria were sonicated, centrifuged and membrane filtered. Materials were stored at -20 °C.

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Antisera against crude haemolysins were prepared by immunizing rabbits of 2.5 to 3.5 kg intravenously by graded doses. After 4–5 doses in 4 day intervals the animals were bled, their sera filtered through membrane filter and stored at -20°C . An antitoxic serum was prepared by the cytotoxic Sephadex G-100 fractions (60 to 70 ml), mixing aliquot amounts with complete Freund adjuvant (Difco) and injecting rabbits subcutaneously, according to the method of CALLAHAN *et al.* [5].

For producing anti-O serum, bacteria heated at 100°C for 30 min, and for anti-OK sera, living bacteria were used. Because of the high anti-haemolytic titres of OK sera prepared by original Hly⁺ strains, an OK serum without increased anti-haemolytic titre was produced by an Hly⁻ EMS-induced mutant of the strain Kreka (designated Kreka/7) used in living state for immunization.

Assay of haemolytic and haemolysin inhibition (neutralization). To the crude haemolysin (0.5 ml) diluted in tubes with PBS, 0.5 ml of washed sheep erythrocytes (2%) was added. Mixtures were incubated at 37°C for 2 hr. The percentual ratio of haemoglobin was determined colorimetrically and the value of 50% haemolysis (HD_{50}) was determined by the linear interpolation method of VON KROGH [6].

Inhibition (neutralization) of haemolysins was performed similarly: mixing the dilutions of sera with 5 to 8 HD_{50} quantities of haemolysins.

Cytotoxicity test and neutralization of cytotoxins. These tests were performed in AV-3 cells (a cell line of human amniotic origin), using microplates, as described previously [1]. The titres of cytotoxins were expressed in the last effective dilution. Neutralization experiments were carried out by mixing serum dilutions with 2 to 4 tissue culture cytotoxic doses of 100% (TCD_{100}), incubating the mixture at 37°C for 30 and at 4°C for 60 min. Values of neutralization were expressed by multiplying the effective dilution with the volume used, calculating for 1 ml of serum.

Lung test and neutralization experiments. The lung test was carried out in BALB/c mice of 10–12 g [1]. Neutralization was performed by mixing 0.025 ml of overnight broth cultures with 0.025 ml of serum dilutions. After incubation at 37°C for 30 and at 4°C for 60 min, the mixtures were instilled into the nostrils of mice superficially anaesthetized with ether. Results were read at 1, 2, 3, 4, 5, 6, 7, 8, 9, 24 and 48 hr. Together with the tests the virulence of the challenging agent was checked and the actual LD_{50} was estimated. The neutralization capacity of sera was expressed in ED_{50} (50% effective dose) and for easier comparison the relative potency (RP) of sera was indicated. The 50% end points (LD_{50} , ED_{50}) were calculated according to KÄRBER [7].

Isolation and characterization of Hly⁻ and R mutants. Hly⁻ mutants were isolated from the strain Kreka by EMS mutagenesis and by plating on blood agar plates [1]. The lack of haemolysin production was checked with the induction method of SMITH [2] and by quantitative assay.

R mutants from the strain Kreka were isolated after EMS mutagenesis by selection with homologous OK serum [8]. Colonies showing spontaneous agglutination in physiological saline were tested further in tube agglutination using saline concentrations from 0.2 to 0.9%. A single clone (R12) was chosen and controlled for O and OK agglutinability. To prevent spontaneous agglutination, bacteria were pretreated with ethanol [9]. All R mutants were tested for sensitivity against the R phages F0, C21, 6SR, FP1, FP3, Ffm, Br2 and Br10 [10] and for lung toxic activity.

Results

Three strains were chosen for detailed investigation. Strain “Kreka” (O4 : K12 : H5) the test object in our previous work [1] had haemolytic cytotoxic and lung toxic activities. Another strain designated 281/54 belonged to the same *E. coli* serogroup and its haemolytic capacity was carried by a Hly plasmid. The third strain designated M-S-15 (O139) was an “oedema disease strain” of porcine origin with a well-expressed haemolytic activity but a rather weak lung toxic capacity.

From these strains crude haemolysins were produced with reliable titres; the HD_{50} values were: Kreka = 1 : 117; 281/54 = 1 : 200 ; M-S-15 = 1 : 1015.

In agreement with earlier observations haemolysin production was the most pronounced in the case of strain M-S-15.

Hyperimmune sera obtained by immunizing rabbits intravenously with these crude haemolysins were tested for haemolysis inhibition (neutralization). The data (Table I) showed that all sera had a high neutralizing capacity for all haemolysins tested suggesting the antigenic identity or close similarity of the haemolysins. Pre-immunization sera too had high anti-haemolytic titres (1 : 20 to 1 : 180). Such natural (?) anti-haemolysins were found in all randomly tested rabbit, human or mouse sera.

Table I

Cross neutralization of the haemolytic activity by antisera produced with crude haemolysins of E. coli strains

Antihemolysins	50% haemolysis inhibition expressed in dilutions of sera (HD ₅₀) against		
	Kreka haemolysin, 4.9 HD ₅₀	M-S-15 haemolysin, 5.3 HD ₅₀	281/54 haemolysin, 8.3 HD ₅₀
anti-Kreka (O4)	1 : 948	1 : 733	1 : 691
anti-M-S-15 (O139)	1 : 751	1 : 1695	1 : 1444
anti-281/54 (O4)	1 : 1488	1 : 1524	1 : 995

Next, the crude haemolysins described above were investigated in AV-3 cell culture for cytotoxicity. Their cytotoxic capacity (titres between 1 : 160 and 1 : 320) permitted to carry out neutralization experiments by the anti-haemolysin sera. According to the data in Table II, all the sera showed a clear-cut cross neutralizing capacity, comparable to the result of the haemolysis

Table II

Neutralization of the cytotoxic effect of crude E. coli haemolysins by anti-haemolytic sera

Sera	Neutralization capacity of sera in units*, against		
	Kreka toxin 4 TCD ₁₀₀ **	M-S-15 toxin 2 TCD ₁₀₀	281/54 toxin 2 TCD ₁₀₀
anti-Kreka haemolysin	2564	666	666
anti-M-S-15 haemolysin	322	322	322
anti-281/54 haemolysin	322	322	322
anti-Kreka cytotoxic fractions	161	322	322

* Neutralization capacity of sera expressed per ml, taking into account the dilution and quantity of serum necessary to reach the neutralization end point

** TCD₁₀₀ = tissue culture dose causing total cell destruction

inhibition test. Another serum prepared by the cytotoxic fractions (Sephadex G-100 column from a concentrated ultrasonic lysate of strain Kreka) had a similar neutralizing capacity.

More technical difficulties arose in testing our sera against mouse lung toxic activity. The "induced haemolysins" had no measurable activity and even the most effective, concentrated ultrasonic lysates were working only in high doses and with delayed lethal effect. This fact may be due to the lower sensitivity of the lung model. The only possibility was to work with living bacteria, though these reduced the proper evaluation of experimental data. An example of this series of experiments is given in the Table III. The challenging

Table III

Neutralization of lung toxicity elicited by E. coli O4 strain 281/54

Sera, ml		Survivors/ infected	ED ₅₀	RP*
anti-Kreka crude haemolysin	0.025	4/4	0.0004	10
	0.0025	4/4		
	0.00025	1/4		
anti-M-S-15 crude haemolysin	0.025	4/4	0.004	1
	0.0025	1/4		
	0.00025	0/4		
anti-281/54 crude haemolysin	0.025	4/4	0.0008	5
	0.0025	2/4		
	0.00025	1/4		
anti-OK (Hly)** serum (by living strain)	0.025	4/4	< 0.00025	> 28.5
	0.0025	4/4		
	0.00025	3/4		

In the experiments 0.025 ml of serum dilution was added to 0.025 ml of broth culture incubated at 37 °C for 30 and at 4 °C for 60 min; 0.05 ml of the mixture was given to mice. Virulence test was carried out in parallel; the LD₅₀ was determined as 1.3 LD₅₀ (about 1.3×10^7 germs)

* Relative potency

** Anti-haemolysin titre, 1 : 7680

agent was the O4 strain 281/54. At a low infecting dose (LD₅₀ = about 1.3), the three anti-haemolysin sera and an OK serum prepared with live bacteria were tested for protective value. All anti-haemolytic sera showed protection (Table III); the homologous (O4) titres were always higher than the heterologous ones. The OK serum gave the highest protective value. These results seemed to suggest that the antibacterial immunity was superior to the anti-

toxic one. Since the OK serum was prepared with live bacteria, the anti-haemolytic titre of this serum was investigated; surprisingly, it was about three times higher (1 : 7680) than that of the best antihaemolytic serum. Therefore, this OK serum, too, had antihaemolysin activity.

In other experiments, when the protective value of O sera was tested, only weak protective titres were found. These experiments did not, however, allow a comparison between antibacterial and antitoxic immunity, because anti-K antibodies might have an important role in inhibiting bacterial growth, consequently fatal lung oedema could not develop. For solving this problem there were two possibilities. One, to produce an OK serum with Hly⁻ mutant

Table IV

*Neutralization of lung toxicity elicited by the R mutant (R12)
of E. coli strain "Kreka" (O4)*

Sera, ml		Survivors/ infected	ED ₅₀	RP*
normal rabbit serum**	0.025	0/4	> 0.025	< 1
	0.0025	0/4		
anti-O, K, Hly*** serum (by living strain)	0.025	4/4	0.00025	30
	0.0025	3/4		
	0.00025	3/4		
anti-O4 serum	0.025	3/4	0.0025	3
	0.0025	2/4		
	0.00025	1/4		
anti-OK serum (Kreka/7 Hly ⁻)	0.025	4/4	0.0025	3
	0.0025	2/4		
	0.00025	0/4		
anti-Kreka crude haemolysin	0.025	4/4	0.00079	10
	0.0025	4/4		
	0.00025	0/4		

The experiment was carried out as in Table III. The LD₅₀ in parallel virulence tests was about 2.5 LD₅₀ (about 3×10^7 germs)

* Relative potency

** Normal rabbit serum containing natural (?) anti-haemolytic antibodies at a titre of 1 : 30

*** Anti-haemolytic antibody content, 1 : 7680

living bacteria, when the serum will not have anti-haemolysin antibodies. Another approach was the isolation of an R mutant with intact toxin producing ability.

Experiments carried out in the mouse lung model showed that an OK serum prepared with a Hly⁻ mutant (Kreka/7) had antibacterial activity, but its level was lower than that of the protection provided by antitoxic sera.

A clone of the R mutant (Kreka R12) isolated as described in "Materials and methods", was tested after ethanol treatment in O and OK sera. Only a rudimentary antigenicity was found, with agglutination titres of 1 : 40 and 1 : 160. The culture showed spontaneous agglutination in 0.2% saline and was sensitive against R phages 6SR, C21, FP1, FP3, Ffm, Br2, Br10 except FO. The wild-type strain was resistant to all the R phages tested. The phage sensitivity pattern of clone R12 seems to characterize an Ra mutant [10].

In Table IV an example of the lung model experiments using this R12 mutant as challenging agent is shown. It is seen that anti-Hly serum had a reliable antitoxic protective value, and only O, K and Hly antisera had a somewhat better effect. O and OK (Kreka/7 Hly⁻) sera, as well as normal rabbit sera, had a very weak or no protective value.

Discussion

On the basis of the experimental data, it was concluded that the toxic, alpha-type haemolysins of *E. coli* are antigens. In agreement with our earlier observation [1], these haemolysins are characterized by a high molecular weight. The antigenic structure of the few haemolysins studied so far seems to be identical. The antigenic identity may also be true for the cytotoxic and lung-toxic substance of the bacterial cell. This may further support our assumption that for the haemolysing capacity, cytotoxicity and lung-toxicity the same molecule is responsible. Nevertheless, this assumption is difficult to prove. The strict correlation between the haemolytic and lung toxic properties of the *E. coli* strains investigated till now, the missing lung and cytotoxicity of the induced Hly⁻ mutants, the loss of these characters by curing the Hly plasmid in case of strain 281/54, and the identity of antigenic structures in cross neutralization experiments, are all supporting the assumption. On the other hand, there are technical difficulties for obtaining further evidence, such as the lack of purified materials, the inability to carry out transduction of the Hly marker due to P1 resistance of the strains, the unsuccessful transfer of the Hly plasmid into cured Hly⁻ clones of strain 281/54. There are other discrepancies among the above-mentioned characters, too. Conditions for haemolysin production are not favourable for the production of the lung toxic substance, and supernatants of high haemolytic activity are without lung toxic effect. This may simply reflect the different sensitivity of the tests. Furthermore, the haemolytic activity is the most sensitive character; its level falls rapidly at room temperature and somewhat slower at 4 °C. Ultrasonic treatment of bacteria destroys

most of the haemolytic activity, but at the same time it releases lung toxic material with a good yield. Such differences in phenotypic characters after all do not contradict the "unitarian theory"; they rather reveal sites of the same molecule with different sensitivity.

We are not certain whether such strains have a role in developing intestinal or extraintestinal diseases. Neither is it known whether toxic haemolysin represents an additive virulence factor. Supposing the pathological role of this toxin, the results of neutralization experiments in the mouse lung model may be informative about protective immunity. It seems that antibacterial immunity, by inhibiting bacterial growth, may offer some protection against toxic manifestations, too. On the other hand, antitoxins having uniform antigenic specificity are effective and they may serve as useful tools in protection.

Taking into consideration the results obtained with the M-S-15 strain of *E. coli* (O139) freshly isolated from swine oedema disease, they warrant a comparative investigation of toxic haemolysin and EDP (Oedema Disease Principle).

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THE EFFECT OF CONTINUOUS HUMAN LYMPHOKINE TREATMENT ON SKIN GRAFT REJECTION IN MICE

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Continuous intraperitoneal treatment with human lymphokines produced by Con-A stimulated peripheral human lymphocytes caused the acceleration of skin graft rejection in mice.

Lymphokines, the biological active products of lymphoid cells in response to antigen or mitogens are considered potential mediators of cellular immunity [1]. Their role manifests in delayed-type hypersensitivity reactions, in protective immunity and in cell cooperation effects [2]. They are products of active *de novo* synthesis [3]. More than twenty factors belong to the family of lymphokines; among these the skin reactive factor, macrophage and leukocyte migration inhibitory factors, mitogenetic, macrophage arming and activating, lymphnode activating and various chemotactic factors (macrophage, eosinophilic, neutrophilic, lymphocyte, basophilic) are regarded to be the most important ones. They have mediator and regulator roles in cell mediated immune reactions, but their immune stimulant features seem to be especially important.

The process of skin graft rejection is regarded to be a special form of cellular immunity where the tissue specific antigens of the graft cells involve the defensive reactions of host lymphocytes and macrophages by the production of soluble lymphocyte products (lymphokines). Especially the activated macrophages have a significant role in the destruction of foreign cells [4–6].

After various suggestions in the literature human lymphokines were produced in suitable quantity and quality for experiments *in vivo* and we have studied whether our preparation accelerated skin graft rejection in mice.

Materials and methods

Animals. Female BALB/c recipient and CBA donor mice weighing 20 g were used. The animals were kept under standard conditions, fed on a standard diet and allowed to drink water *ad libitum*.

Preparation of lymphokines. Normal human blood anticoagulated by citrate was used. Preparation of lymphocytes was carried out in Uromiro–Ficoll solution in Parker's 199 medium at room temperature. No serum was added. The samples were centrifuged at 800 g for 15 min and washed twice; they contained 90–95% mononuclear cells.

Lymphocytes (10^7) were cultured at 37°C for 36 hr in 5% carbon dioxide and 95% air in the presence of 30 μ g concanavalin-A (Con-A, Sigma) completed with 100 IU penicillin and streptomycin per ml.

The cell-free supernatants were chromatographed on Sephadex-G 75 column using 0.01 M phosphate buffer pH 7.4 for elution. In the control preparation, Con-A was added at the end of cultivation. The protein containing fractions were collected and concentrated with Amicon B-15. Protein concentration of the preparations was generally about 0.2–0.3 mg/ml.

The leukocyte migration inhibitory factor and skin reactive factor [7] containing preparations were termed active supernatants (AS) and controls (CS). Leukocyte migration inhibitory factor activity was tested with human leukocytes according to KINCSES and SZABÓ [8]. Skin reactive factor testing was carried out in guinea pig skin by measurement of the blue areas on the inner surface of the skin after intravenous injection of 1% Evans blue, 4 hr after an intradermal injection of 0.2 ml CS or AS.

Skin transplantation. Under pentobarbital anaesthesia, the tail skin of CBA femal mice was prepared, cut in 5 × 7 mm pieces and kept in Parker's 199 medium completed with antibiotics.

The back of the recipient BALB/c female mice was shaved and a skin window was cut by scissors to the papillary layer of the corium. The graft was placed in the skin window so that a 1 mm gap remained between the graft and the edge of the skin, and after sprinkling with penicillin and streptomycin it was fixed by a bandage. Two days before the expected day of rejection, on the 8th day, the bandage was removed and the state of the graft was registered daily.

Lymphokine treatment. Fifteen grafted BALB/c mice were injected intraperitoneally with 0.2 ml of the human lymphokine preparations and their controls every second day, all in all 5 times until the 8th day.

Statistical analysis. The standard deviation (SD) of the values from the averages was determined; then Student's *t* test (after Fischer) was applied for estimating the differences between the graft-rejection days [9].

Results and discussion

Table I shows that the treatment of mice with AS had significantly accelerated skin graft rejection, from 12.4 days in the controls to 10.3 days.

Although YOSHIDA and COHEN [10] found that *in vivo* intravenous lymphokine treatment in tuberculin hypersensitivity one day before challenge decreased the inflammatory reaction by causing a remarkable loss of monocytes in peripheral blood, in the present experiments the immuno-stimulating effects of lymphokines, especially their lymph node and macrophage activating effects manifested during the 8-day treatment. The graft rejection accelerating effect was significant but not strong.

Table I
*Effect of human lymphokine treatment
on skin graft rejection in mice*

Groups of animals	No. of animals	Rejection time (days) mean $\pm$ SD
Control (CS)	15	12.4 $\pm$ 0.8
Lymphokine (AS)	15	10.3 $\pm$ 0.8*

* $p < 0.001$

To explain these processes, we have to consider the possibility of a slight immunization of mice against the human lymphokine molecule during treatment, although the lymphokines are not good antigens and the mouse is not a species sensitive to immunization. Still, the immunostimulating effect of lymphokines influencing the peripheral lymph organs in the early and middle phases of treatment seems to be responsible and sufficient for the graft rejection accelerating capacity.

These data support the view that lymphokines, beside their local pathogenic role in inflammatory and immune processes, might have a more general, permanent, eventually physiological significance in the regulation of cellular immunity [11].

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TYPES OF THE “IRREGULAR” ORNAMENTATION OF THE SURFACE SHEATH OF STREPTOMYCETE SPORES AND AERIAL HYPHAE

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Irregularities in the structure and ornamentation of the surface sheath of aerial hyphae and spores, a neglected phenomenon, are widely distributed among the members of the genus *Streptomyces*. Several frequent types of these morphogenetic irregularities, and a certain regularity in the appearance of abnormalities, have been described. An important component of the sheath material, the presence and quantity of which may determine the level of transformation from smooth into weakly or even strongly ornamented surface cover, or a probably subsequently activated hypothetical transformation inducing agent produced in sufficient or insufficient amounts or in adequate or inadequate form, more or less synchronously with the fibrous sheath material by the tip-cell unit of growing aerial hyphae, should be responsible for the morphogenetic changes taking place in the sheath after the termination of hyphal growth.

Since nearly 25 years ago FLAIG *et al.* [1] have shown that certain *Streptomyces* species produce spores with a warty, spiny or hairy surface, a number of electron micrographs were prepared on streptomycete sporophore materials for taxonomic purposes. The spore silhouette, which is due to the presence or absence of ornamentation of a very thin outer surface sheath, is now considered one of the most reliable taxonomic criteria of streptomycetes [2], although in some cases differently ornamented and also smooth spores may be observed in the sporophores of the same strain [3]. Unfortunately, the different types of “abnormalities” in sheath ornamentation, which incidentally may cause difficulties in taxonomic work, were neglected during many years of the fine structure research work on streptomycetes. According to some workers [4], the lack of surface ornamentation on a few spores among ornamented ones may partly be due to the partial or complete disruption of the ornamented sheath material. SZABÓ *et al.* [5] and SZABÓ [6] who studied the abnormalities in the transformation of surface sheath from the original smooth into ornamented one, in *Streptomyces coeruleorubidus* have demonstrated that the occurrence of smooth spores among ornamented ones within the same sporophore, the frequently observed differences in the density and size of surface ornaments on individual spores, as well as the transformation of sheath material not only on the sporulating hyphae but also on the surface of the sterile stalk, are not randomly occurring phenomena. These “irregularities” presumably mirror the malfunctioning of an unknown regulatory mechanism acting along the whole length or a long segment of hyphal filaments. The tip-

cell unit of growing hyphae may produce a sheath transformation inducing agent [6], acting more or less independently of the spore formation processes. Troubles in the production and activities of this hypothetical agent may cause the appearance of irregular spore silhouettes and sheath ornaments on the sterile aerial hyphae. According to BLUMAUEROVÁ *et al.* [7], the developmental mutant *spo-2* of *S. coeruleorubidus* which produces, in contrast to the wild type, spores covered with short rudimentary spines, may partially be blocked in the formation or activity of the regulating agent.

The purpose of the present study was to demonstrate the most common and characteristic types of abnormal or irregular sheath ornamentation and their frequency among the members of the genus *Streptomyces*, furthermore to find some regularity in the appearance of sheath transformation abnormalities by studying the development of the sheath of complete, intact sporophores, long spore chains and of long aerial hyphal filaments.

Materials and methods

To observe the local irregularities and quantitative differences in transformation of the original smooth sheath into ornamented one on the surface of the individual aerial hyphal filaments during sporulation, whole mount preparations of sporophore materials were prepared and the silhouette of complete sporophores was examined electron microscopically. Coated grids were touched gently to the surface of the sporulating aerial mycelium of the examined strains grown on agar plates of different composition, in Petri dishes. The material mounted on the grids was examined and photographed after drying at room temperature. Whole mount preparations were examined and photographed partly in a UEMV-100 B, and partly in a TESLA electron microscope. In the electron microscopical laboratories of the Agrochemical and Pedological Research Institute and of Eötvös L. University, more than 500 *Streptomyces* strains, among them more than 200 authentic, mostly ISP and ATCC strains were examined.

Results

On the basis of direct electron microscopic observations and of the subsequent study of a large number of electron micrographs deposited in the collection of our laboratories it has been concluded that "irregularities" in sheath ornamentation are widely distributed among streptomycetes with a spiny or hairy spore surface. Besides, several characteristic types of these irregularities can be recognized which occur at different frequency with the individual strains of different species. The most common types of these irregularities which represent the results of abnormal pathways of submicroscopic morphogenesis, are as follows.

(1) The sheath of the sterile stalk of streptomycete sporophores which during sporulation processes remains generally smooth may, sometimes, become ornamented when on the proximal end of the spore chain, the ultimate spore,

is still ornamented (Plate I, Figs 1, 2, 3, 4). If the latter is smooth, the surface sheath of the sterile stalk does not bear any ornament (Plate II, Fig. 1).

(2) In certain cases, transformation of the smooth sheath of sterile aerial hyphae into a spiny or hairy ornamented surface cover can be observed, without any sign of differentiation and development of spores in these fila-

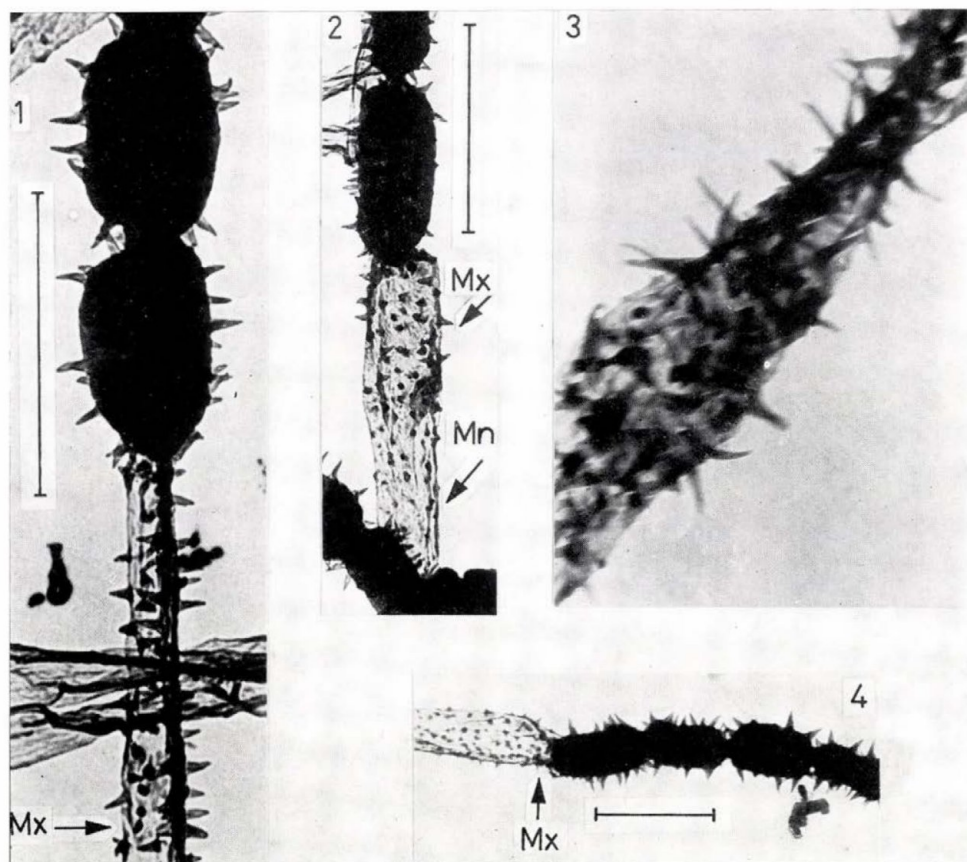


Plate I. Figs 1-4. Electron micrographs of the proximal ends of streptomycete sporophores (air dried whole mounts). Bar = 1.0 μ m. 1. *S. coeruleorubidus* FBUA 328; 2. *S. coeruleorubidus* FBUA 328; 3. *S. hirsutus* ISP 5095; 4. *Streptomyces* sp. B/A-10

ments (Plate III, Fig. 2). Studying the silhouette of the sterile aerial hyphal filaments in certain cultures two types may be observed, filaments with smooth sheath, and filaments with ornamented sheath.

(3) There frequently exists along the sporulating hyphae a decreasing tendency in the intensity of transition of the smooth surface sheath to a spiny or hairy ornamented one. It may be measured either directly from the tip-spore at the distal end of the sporophore or, more frequently, from one of the intermediate members of the spore chain (Plate II, Figs 1, 2, 3, 4; Plate III,

Figs 3, 5; Plate IV, Fig. 1), but always towards the sterile stalk. The density and size of sheath ornaments on the tip-spore are generally identical with those of the most ornamented spores of the chain. If the sporophore contains only one ornamented spore in the chain, then this is exactly the tip-spore (Plate III, Fig. 4). In the case of mature sporophores, if the tip-spore is smooth, the whole

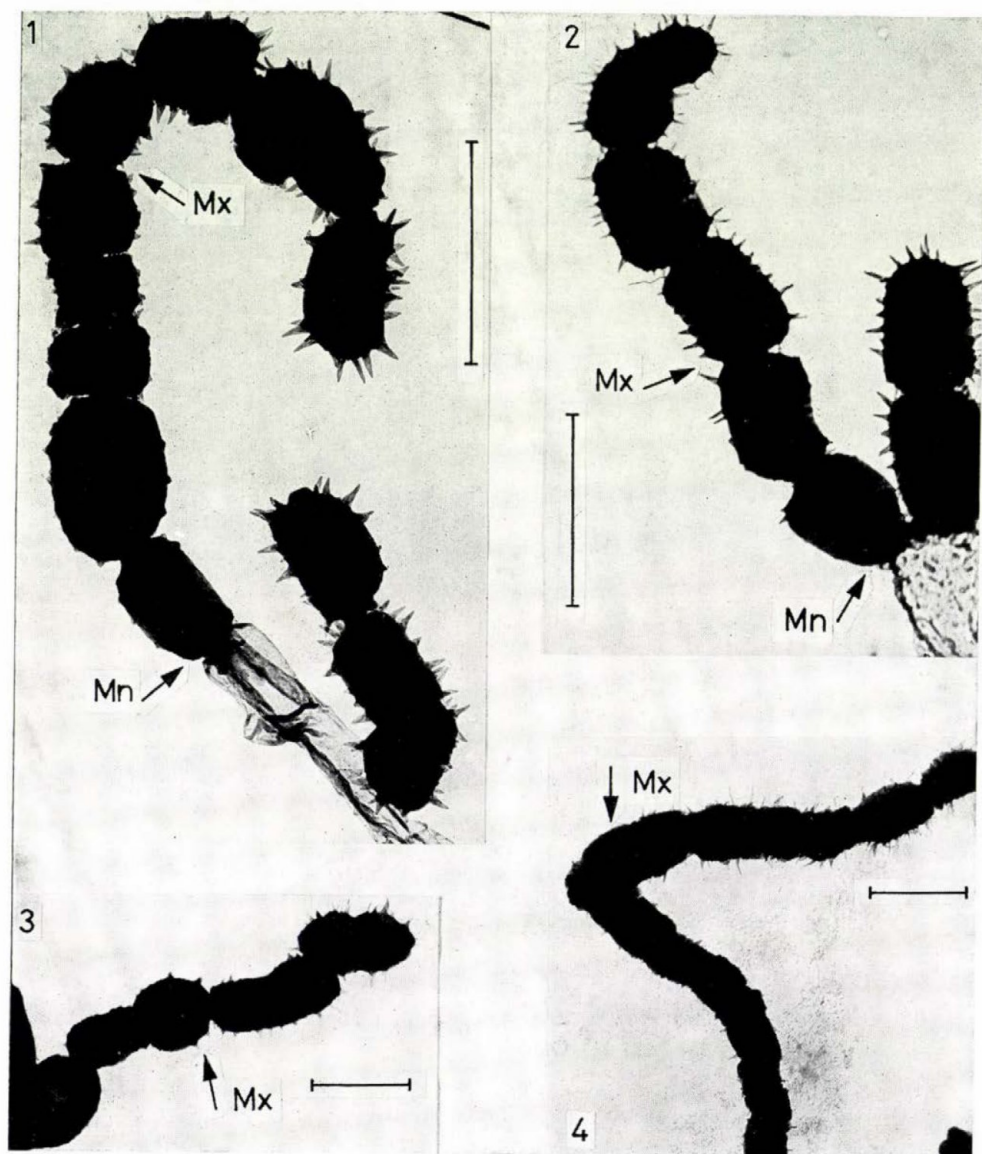


Plate II. Figs 1-4. Electron micrographs of complete streptomycete sporophores (air dried whole mounts). Bar = 1.0 μ m. 1. *S. coeruleorubidus* FBUA 328; 2. *S. chartreusis* 2-6; 3. *Streptomyces* sp. B/A-10; 4. *S. griseoflavus* CBS Cifferi

spore chain and the sterile stalk are also smooth. That member of the decreasingly ornamented spore chain, which as ultimate one still bears spiny or hairy rudiments, indicates the local end-point (Mn; Plate V) of the transformation processes acting along the sporophore sheath. The distance between the tip-

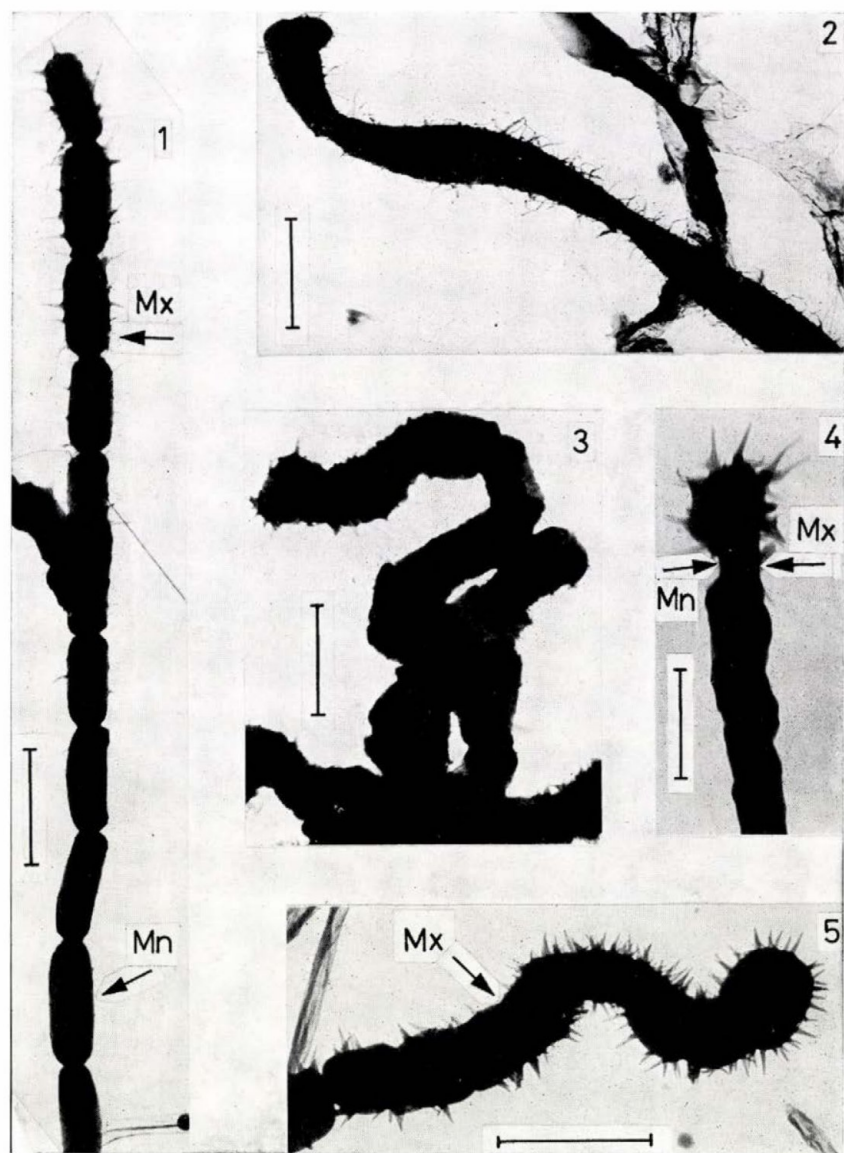


Plate III. Figs 1-5. Electron micrographs of streptomycete sporophores and aerial hyphae (air dried whole mounts). Bar = 1.0 μ m. 1. *Streptomyces* sp. 2-49 (Series Lavendulae); 2. *Streptomyces* sp. 672; 3. *Streptomyces* sp. 4169; 4. *Streptomyces* sp. 2/i; 5. *Streptomyces chartreusis* 3-12



Plate IV. Fig. 1. Electron micrograph of a segment of an asporophore of *Streptomyces* sp. S-3-3. Fig. 2. Short, rudimentary spines on the sheath of spores of *Streptomyces* sp. 2-30-0 (air dried whole mounts)
Bar = 1.0 μ m

spore and this ultimately ornamented spore (Ts-Mn; Plate V) is very different, and this segment of the hyphae may involve quite different members of spores. Generally, the tip-spore is followed by subsequent spores which are superimposed with a sheath whose ornamentation is nearly equal to that of the tip-spore, at about the possible maximum. That spore which is located in the spore chain furthest away from the tip-spore but is still maximally ornamented (Mx; Plate V) indicates the local end-point of the maximally acting sheath-transformation processes and at the same time the starting point of a hyphal section (Mx-Mn; Plate V), the outer sheath of which may be characterized by a gradually decreasing intensity of molecular re-arrangements.

(4) If the sheath transformation is extending to the sterile stalk of sporophores, or the sterile hyphal filaments are also ornamented, the density and

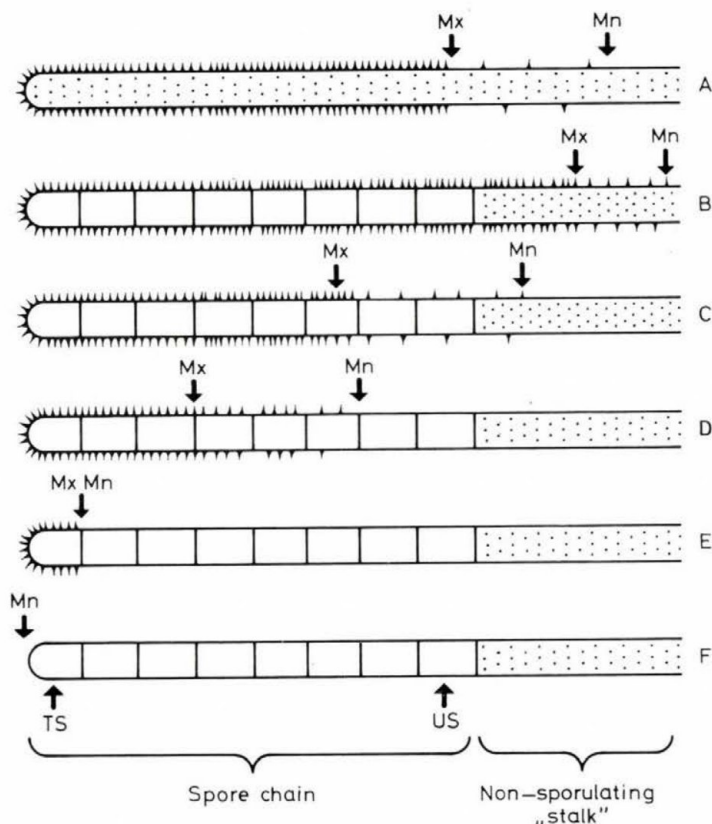


Plate V. Schematic representation of some types of irregular surface ornamentation of the hyphal (A) or sporophore (B-F) sheath of *Streptomyces* spp. characterized by production of both (spiny or hairy) ornamented and smooth spores. Mn = at this point a minimum sheath ornamentation is still detectable. Mx = at this point the sheath ornamentation is still at the maximum but then begins to decrease. TS = tip-spore at the distal end of the sporophore. US = ultimate member of the spore chain

size of ornaments may, on these too, show a gradient of decreasing intensity from the distal towards the proximal region (Plate I, Fig. 2). Accordingly, the sterile stalk or the sterile aerial hyphal filaments may be maximally ornamented along their distal segments, and the two cardinal points of sheath ornamentation (Mx and Mn) can also be recognized on them (Plate V).

(5) Concerning the size of ornaments on the envelope of sporophores, there exist two particular varieties of ornamented spore chains which occur either mixed one with the other and with the normal type in the same culture of a strain, or distinctly and exclusively in the aerial mycelium of selected strains. (a) The size of ornaments (spines or hairs) is approximately the same along the whole length of the sporophore, but considerably smaller or shorter (sometimes only rudimentary) than that of the normal ones on the sporophores of typical strains of the species (Plate IV, Fig. 2). (b) Similar to (a), but the ornaments are considerably stronger or longer than those on the normal type sporophores.

Discussion

During the process of sporulation, the outer fibrous sheath of hyphae is known to be transformed drastically by the elaboration of spines or hairy ornaments and, in addition, to break up into spore size segments that cover each spore as a mantle [4, 5, etc.]. Sporulation processes acting within the hyphal wall are so closely correlated with sheath transformation that according to some workers [8] unsegmented hyphae bearing spines, hairy appendages or their rudiments represent hyphae undergoing sporulation.

These "irregularities" clearly demonstrate that the process of sporulation is not strictly synchronized spatially with the molecular arrangements and re-arrangements (formation of surface ornaments) occurring outside the wall of the sporulating hyphae in the sheath. SZABÓ [6] has suggested that the tip-cell unit of growing hyphae probably produces a sheath-transformation inducing agent (or agents) acting more or less independently of the spore formation processes. There seem to be two alternatives: (1) The agent is produced after completion of hyphal growth and spreads subsequently from the tip along the fertile hyphal section towards the sterile stalk. The hyphal concentration of the agent is highest at the tip, and between the tip and the Mx-point it may be locally greater than required to induce a maximum possible ornamentation. The Mx-point may be considered a point of inversion. From here up to the Mn-point the agent's concentration or transforming capacity decreases gradually to zero. (2) The tip-cell unit of aerial hyphae, which is continuously producing the fibrous sheath material during its growth, may synchronously produce, but only after reaching a certain length of the hypha, the transforming agent in an inactive form. Production of the agent would gradually increase,

but after reaching the level potentially capable of inducing maximum ornamentation, would persist at this level without any fluctuation. Activation of the agent would then follow either after completion of growth, or at the beginning of sporulation.

On the basis of the findings of STRUNK [9] we know that, at least in the case of *Streptomyces melanochromogenes*, in the spore chains the delimited spore compartments are temporarily connected by two centrally located septal structures, named nozzles, which are perforated by "microplasmodesmata". These septal pores ensure the communication between the neighbouring cells and allow intercellular connections between the adjacent spore compartments during the process of maturation. It is important that the development of spines on the surface sheath begins before the reduction of these perforated septa and the disturbance of intercellular connections.

It is also possible that the supposed regulatory agent is simply a building component of the surface ornaments. If the component is lacking, transformation of the sheath from smooth into ornamented does not take place. When the tip-cell unit produces the component permanently but at a very low level, the ornamentation will be uniformly rudimentary. All changes in the production would cause the appearance of gradients in the density and size of ornamentation.

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AN ALTERED HEAT-LABILE ENTEROTOXIN (LT') PRODUCED BY ESCHERICHIA COLI SEROGROUP O55 STRAIN*

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An *Escherichia coli* serogroup O55 strain produced heat-labile enterotoxin only, which exerted unusual effects on cell cultures; it caused elongation of CHO and HeLa cells but no changes in Y-1 cells. Injection of this substance, designated LT', into mouse foot pad and rabbit skin caused a well-expressed necrotic effect beside the LT-like activity. LT' showed no cytotoxic effect and failed to produce mouse lung oedema. The strain was not haemolytic. According to Sephadex G-100 fractionation, the LT' had a high molecular weight. The LT' and the necrotic activities could not be separated by fractionation. Neutralization experiments suggested an antigenic relationship between LT and LT'. The antigenic deficiency of LT' was closely related to the common antigenic component of LT and cholera toxin. The necrotic effect caused by crude LT' was neutralized only by the homologous serum.

Escherichia coli strain O55 was obtained from Dr. B. ČERNA (Institute of Microbiology of the Czechoslovak Academy of Sciences, Prague); its enterotoxigenicity was studied in rabbit loop dilatation test [1]. Investigating the toxigenicity of the strain in cell cultures, we found that it caused a characteristic elongation of CHO cells, without rounding of Y-1 cells. In their recent paper TAKEDA and MURPHY [2] reported that an enterotoxin causing elongation of CHO cells only was informed by a temperate phage.

Herewith we present our data on the characterization of this LT, which was designated LT'. It was found to be inducible by mitomycin C. The possibility of the information of this type of toxin by a temperate phage is under investigation.

Materials and methods

Strains. *E. coli* O55 Ent⁺ (Černa), as well as *E. coli* No. 263 LT⁺ST⁻ (O8: K87, K88a-b: H19) were used.

Medium and cultivation. For enterotoxin production a modified Sakazaki medium [3, 4] in which the Myosat was substituted by Levinthal broth, was used. Cultures were shaken in Psycrotherm (New Brunswick) at 37 °C for 18 hr.

Mitomycin C induction was carried out in media containing 2 µg/ml mitomycin C (Kyowa, Tokyo) [2].

Concentrated crude enterotoxin was prepared as described earlier [4, 5] by ultrasonic lysis, dialysis, membrane filtering, and freeze-drying. Materials were then dried over P₂O₅ and distributed in vials filled with N₂ and stored at -20 °C.

As toxin control dry cholera toxin (prepared by Dr. R. A. FINKELSTEIN, Lot No. 4493 G) with a nominal value of 1800 Lb/ml was used (CT).

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Molecular filtration was performed on a Sephadex G-100 column. For dissolving materials of 200 mg and as eluent Tris-HCl (0.005 M) containing EDTA (0.001 M) buffer pH 7.5 was used. Fifty fractions of 5 ml each were collected and stored at -20°C .

Sera. A cholera antitoxin (Swiss Serum Institute, Bern) with a value of 36 000 loop U/ml and 10 000 PF U/ml (horse IgG), as well as an antitoxic serum prepared by the partially purified LT of strain 263 were used. A similar serum was prepared against LT', following the method of CALLAHAN et al. [6].

Assay of enterotoxigenic activity. ST activity was tested in 3 days old suckling mice, according to JACKS and WU [7].

LT activity was measured in the mouse foot oedema test [8] using one of the hind legs of mice. Readings were made after 48 hr on the basis of the average oedema fluid weight, calculated from the weight differences of inoculated and control hind pads. This was expressed in mg and also in biological units [9] taking 0.75 mg oedema weight as one biological unit. LT activity was assayed with the blueing test on rabbit skin according to EVANS et al. [10]. Eighteen hours after the intradermal inoculations, Pontamin Sky Blue (Serva) at a concentration of 5% was injected intravenously in a ratio of 1.2 ml/kg rabbit. Readings were made after one hour.

Cytotoxic and cytotoxic effects were investigated in cell cultures (Y-1, CHO, HeLa, AV-3) by micromethods [5].

Neutralization experiments were carried out in mouse foot oedema test, blueing test and in CHO cells. In all cases dilutions of antisera were mixed with equal volumes of enterotoxins diluted to a value found optimal in preliminary experiments. These mixtures were incubated at 37°C for 30 min and at 4°C for one hour.

Mouse foot oedema test and blueing test were evaluated as in toxin titration. Both tests served also for the neutralization of the necrotic effect. On rabbit skin the blueing reaction and the necrosis did not disturb reading the results. Neutralization experiments were carried out in CHO cells [5].

Results

1. *Characterization of the crude enterotoxin.* Since repeated experiments showed that *E. coli* O55 (Černa strain) produced a heat-labile enterotoxin having a cytotoxic effect in CHO but not in Y-1 cells, we have attempted to characterize this toxic substance in detail. This unusual effect of O55 enterotoxin was exerted both by culture supernatants and by ultrasonic lysates. Addition of $2\text{ }\mu\text{g/ml}$ mitomycin C to the media increased its cytotoxicity for CHO cells 8 to 16 times.

From the Černa strain a concentrated (freeze-dried) ultrasonic lysate was prepared and its ST and LT activities were tested. In suckling mice no dilatation was observed; even $4000\text{ }\mu\text{g}$ crude toxin was found to be ineffective, while in the blueing test $0.73\text{ }\mu\text{g}$ still caused blueing (about 1 BD). The same amount had, in addition, a marked necrotic effect which inhibited the development of oedema in the mouse foot test. This somewhat paradoxical reaction is presented in Table I.

Concentrated crude enterotoxin was tested also in CHO, Y-1, HeLa and AV-3 cells. HeLa and AV-3 were used to demonstrate a cytotoxic reaction expected on the basis of necroticity. According to our earlier observation [11], a toxic haemolysin of *E. coli* showed necroticity as well as cytotoxicity. The results obtained in cell cultures are summarized in Table II.

These data showed clearly that Y-1 cells are not sensitive to the toxin, which induced a clear-cut elongation of CHO cells. No cytotoxic reaction was

observed, but surprisingly in young HeLa cells an elongation-like reaction was shown. Dry cholera toxin and crude LT of *E. coli* strain 263 had a cytotoxic effect in both Y-1 and CHO cells. The cytotoxic effect of O55 LT¹ demonstrated in CHO culture proved to be heat labile, like in the other LT specific tests. (The necrotic principle was also heat labile.)

Table I

BALB/c mouse foot oedema test with freeze-dried ultrasonic lysate of E. coli O55 (strain Černa). Inhibition of oedema development by the necrotic effect

Dose, µg	Oedema weight		Necrosis
	$\bar{x}$, mg	BU*	
2000	57.5	0.7	++++
1000	137.0	1.8	++++
500	167.5	2.2	+++
250	220.0	2.9	+++
125	230.0	3.0	+++
1000 (100 °C, 30 min)	45.0	0.6	—

* One biological unit means 0.75 mg of oedema; positive reaction over 1 biological unit

Table II

Activity of the freeze-dried ultrasonic lysate of E. coli O55 (Černa) in cells

Enterotoxins	Activity in cells (reciprocal titres)			
	CHO	Y-1	HeLa	AV-3
O55 LT ¹ (native)	128 ²	— ³	128 ²	—
O55 LT ¹ (100 °C, 30 min)	—	—	—	—
263 LT (native)	128	128	—	—
Cholera toxin (4493 G)	512	512	—	—

¹ Provisional designation

² Elongation without any cell degeneration

³ Negative in 1 : 4 dilution

The marked necrotoxicity was in contrast to the effect of the haemolysin [11] which had no cytotoxicity, no haemolytic activity and no lung toxicity.

2. *Sephadex G-100 fractions.* Two hundred mg of freeze-dried crude toxin of *E. coli* O55 Černa were fractionated on Sephadex G-100 column. Fractions

were tested in CHO and AV-3 cells and for blueing and necrotoxicity in rabbit skin (blueing) test. Data are summarized in Table III.

Table III
Activity of Sephadex G-100 fractions of E. coli O55 (Černa)

Fractions		Reciprocal titres or activity in cells of		Rabbit blueing test	
number	volume, ml	CHO	AV-3	blueing, mm ²	necrosis, mm ²
1-11	0-55	—*	—	—	—
12-14	55-70	32-64	—	94-132	94-153
15	70-75	128	—	132	113
16	75-80	256	—	113	113
17-18	80-90	128	—	113	113
19-20	90-100	64	—	50-78	38-63
21-22	100-110	16-32	—	19-28	12-19
25-50	110-250	—	—	—	—

* Negative reaction; in cell cultures negative in a dilution of 1 to 4; in blueing test under 12 mm² of blueing or necrotic area

Table IV
Cross neutralization experiments with LT^r, LT, CT and their antitoxic sera in mouse foot oedema test

Antitoxin	Amount, ml	O55 LT ^r toxin (100 µg)			263 LT toxin (125 µg)		CT (Lot 4493 G) 1.5 Lb	
		mg oedema	BU*	necrosis**	mg oedema	BU*	mg oedema	BU*
anti-LT ^r (O55)	0.025	0	0	—	47.5	0.63	145.0	1.93
	0.0025	10.0	0.13	—	65.0	0.86	102.5	1.36
	0.00025	62.0	0.82	+	82.5	1.10	110.0	1.46
anti-LT (263)	0.025	12.5	0.20	+++	17.5	0.23	100.0	1.33
	0.0025	62.5	0.80	+++	22.5	0.30	57.5	0.76
	0.00025	110.0	1.40	+++	50.0	0.66	77.5	1.03
anti-CT (Swiss Serum)	0.025***	83.0	1.10	+++	35.0	0.46	17.5	0.23
	0.0025	102.0	1.36	+++	27.5	0.36	2.5	0.03
	0.00025	105.0	1.40	+++	52.5	0.70	2.5	0.03
Control (without serum)		100-125	1.3-1.6	+++	166.0	2.21	122.0	1.62

* 1 biological unit = 0.75 mg of oedema

** —, +, +++ degrees of necrosis (nil, light and severe)

*** Cholera antitoxin originally diluted to 1 : 20; 0.025 ml means 1 : 20 dilution

Numbers expressing the values of biological units are printed in italics in case of positivity

It is seen that the cytotoxic effect on CHO cells went parallel with the blueing and necrotic activity. LT-like and necrotic activities seem to be inseparable by Sephadex G-100 fractionation.

3. *Neutralization experiments.* Cross neutralizations were carried out in mouse foot pad, rabbit blueing test and CHO cells. Sera were tested against standard LT and O55 LT (designated LT') as well as cholera toxin (CT).

(a) *Neutralization in mouse foot oedema test.* In this model, though only qualitatively, the rate of neutralization can be measured by preventing the development of necrosis. Results are presented in Table IV which shows a cross neutralization between LT and LT' in respect of the development of oedema (enterotoxic effect). Neutralization was, however, more effective in homologous than in heterologous system, there was no protection against heterologous toxins when a minimum quantity of serum (0.00025 ml) was used. There was a striking difference in the effect of cholera antitoxin against LT and LT'. While standard cholera antitoxin neutralized LT completely, it had no effect on LT'. In reciprocal experiments neither anti-LT nor anti-LT' neutralized CT (Table IV), only the undiluted anti-LT serum offered some protection.

The necrotic effect of LT' was neutralized only by the homologous serum (Table IV).

(b) *Neutralization in rabbit blueing test.* This test beside evaluating PF (Permeability Factor) neutralization permitted a quantitative assay of the neutralization of the necrotoxin. Results are summarized in Table V. Data provided by this model were in good agreement with those of the mouse foot oedema test. There was a cross neutralization between LT and LT', but homologous titres were higher than heterologous ones (640 vs. 80 and 640 vs. 160, respectively). While cholera antitoxin neutralized the LT effectively, a high concentration of anticholera toxin was needed to neutralize LT' (640 vs. 10). Contrary to the mouse foot oedema test, there was a weak neutralization against LT', which may be attributed to the higher sensitivity of the blueing test. Anti-LT and anti-LT' antitoxic sera did not neutralize CT; this is in agreement with the mouse foot oedema test, and also with our earlier findings [12]. It is possible that the antigenic relationship is not unilateral, and that sera produced by crude LT preparations have low antitoxin titres.

The necrotic effect of LT' material was neutralized only by the homologous, anti-LT' serum (Table V).

(c) *Neutralization in CHO cells.* Results of cross neutralization experiments carried out in CHO cells are summarized in Table VI. The data obtained were in good agreement with the results of the mouse foot oedema and rabbit blueing tests. There was an antigenic relationship but no identity between LT and LT', because homologous antitoxic sera have a definitely higher neutralizing effect. The common antigenic factor of LT and CT might not be present

Table V
Cross neutralization experiments with LT', LT, CT and their antitoxic sera in rabbit blueing test

Antitoxins	Reciprocal neutralization titres against toxins of			
	O55 LT'	(~1 BD)*	263 LT (~1 BD) blueing	CT dry (~2 BD) blueing
	blueing necrosis			
anti-LT' (O55)	640	320	80	— **
anti-LT (263)	160	—	640	—
anti-CT (Swiss Serum)	10	—	640	1280

* 1 BD (blueing dose) = about 50 mm² area

** Negative in a serum dilution of 1 to 10

Table VI
Cross neutralization experiments with LT', LT, CT and their antitoxic sera in CHO test

Antitoxic sera	Reciprocal neutralization titres against		
	O55 LT' 1.5 TCD ₃₀ *	263 LT 2 TCD ₃₀	CT (dry) 2 TCD ₃₀
anti-LT'	640	160	< 10
anti-LT	16	128	< 10
anti-CT***	4	128	640

* TCD₃₀ = tissue culture (elongation) dose of about 30% of cells

** Swiss Serum at 1 : 20 dilution

in LT', because while the neutralization of LT by cholera antitoxin is expressed, it is very low or missing in the case of LT'. Antitoxic sera produced by LT and LT' were ineffective against CT in CHO cells.

Discussion

In contrast to the more or less clarified structure of cholera toxin, our knowledge is rather limited regarding the molecular structure of the heat-labile enterotoxin produced by *E. coli* strains. FINKELSTEIN *et al.* [13] suggested that LT was composed of a single polypeptide. This substance is fragmented into 20 000 to over 100 000 dalton molecular weight active fragments by proteolytic cleavage during production and purification. Molecular composition of LT is currently under investigation by DORNER [14].

At any rate it was shown by VAN HEYNINGEN [15] that a sialidase resistant ganglioside (G_{M1}) serves as receptor for CT. The receptor binding part of the CT is the so-called B-region [16]. It seems that the G_{M1} serves as receptor for both CT and LT, an assumption supported by the fact that G_{M1} inhibits both CT and LT binding to CHO cells [17], and also inhibits the steroid generating properties of the toxins (both CT and LT) in Y-1 and OS₃ cells [18]. Furthermore, no enhanced cytotoxic effect was observed [19] when CT and LT were simultaneously added to Y-1 cells. Cross neutralization experiments supplied further information though not without contradictory results. Mostly partial neutralization was described [17, 20, 21] when antisera to CT and LT were cross tested with the toxins. Since it is believed that neutralizing antibodies are directed against the receptor binding B-region of CT [22], studies with cholera-genoid (B-region of CT) are certainly of importance. The separated B-region inhibited the cytotoxic effect of CT, but not that of LT in Y-1 cells [23]. Similar results were obtained in CHO cells by GUERRANT and BRUNTON [17]. These data indicate perhaps that the B-region of CT might not be identical with the receptor binding region of LT. On the other hand, purified A-region of CT resulted in a cytotoxic effect in high doses [24].

Study of LT' may be useful for clarifying the relationship between CT and LT, and also the structure of LT. LT' differs from classical LT in causing cytotoxic effect only in CHO, but not in Y-1 cells. Heat-labile enterotoxins are known to act through cAMP as the mediator, which in case of Y-1 cell leads to a functional change, namely the synthesis and secretion of $\Delta^4,3$ -keto-steroid. No such data are available for functional changes in CHO or other sensitive cells. SPEIRS *et al.* [25] found that active fragments of LT during purification were effective for Y-1 (and Vero), but not for CHO cells. This may indicate that there is a difference between "elongation" and "rounding" related to the particular metabolic pathways of CHO or Y-1 cells.

The results of our cross neutralization experiments suggest an a,b-a,c-like antigenic relationship between LT and LT'. Furthermore-they point to the possible antigenic defect or modification of LT'. This defect seems to be the common antigenic component of CT and LT. The antigenic change may suggest a change in the receptor binding region of LT', but our data do not permit to draw such conclusions.

By partial purification of LT' with Sephadex G-100 fractionation the necrotoxin could not be separated from LT'. The fact that anti-LT serum had no inhibitory effect on the development of necrosis suggests the existence of an independent necrotoxin molecule. This necrotoxin has no relationship with the toxic haemolysin described by us [11]. Strain *E. coli* O55 Černa has no haemolytic capacity or lung toxicity. The crude toxin has no cytotoxicity and it could not be neutralized in the mouse foot oedema test by the antiserum to toxic haemolysin.

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In memoriam



Lóránd Kesztyűs

Professor Lóránd Kesztyűs, Director of the Institute of Pathophysiology, University Medical School, Debrecen, Deputy Chairman of the Medical Division, Hungarian Academy of Sciences, died on August 17, 1979 at the age of 65.

He was born in Sarkad, Hungary on April 11, 1915. He entered the Medical Faculty of the Debrecen University in 1932 and graduated in 1938. He began to work already in 1934 in the Institute of Physiology and Pathophysiology under the guidance of Professor I. Went, and became known for his research in immunopathology. His theoretical grounding was greatly improved by a one-year fellowship at the Institute of Pharmacology, University of Berlin. As an appreciation of his outstanding abilities, he was appointed associate professor at the age of 28, and in 1948 he became Director of the Institute of Pathophysiology. During his professorship, as a very competent scientist, he developed a new field of research on immunology and immunopathology. His juniors contributed to the development of immunology in several institutes in this country. He wrote more than 160 papers, published the monograph "Allergy" in cooperation with B. Fornet, the book "Immunität und Nervensystem", several Hungarian editions of a text-book on pathophysiology and many chapters in different books. He was a member of the Board of the Hungarian Society of Physiology, and Hungarian Society of Microbiology, founder

and life Chairman of the Hungarian Society of Immunology, member of the Editorial Board of *Acta Microbiologica*. In 1967 he was elected as corresponding, in 1976 as ordinary member of the Hungarian Academy of Sciences, in 1976 as Deputy Chairman of the Medical Division of the Academy. During four decades he was a tireless and competent teacher and was liked and respected by his students and by all those with whom he came in contact. He was first Dean and Rector of the University Medical School of Debrecen for more than 15 years. His outstanding public life activities combined with his friendly personality made him welcome everywhere. He was several times honoured with the highest decorations and in 1978 with State Prize.

With the passing of Professor Lóránd Kesztyűs, we have lost an outstanding authority in theoretical medical research. He will be remembered by his colleagues and his scientific life-work will be continued by a great number of his students.

LAJOS VÁCZI

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NATURAL ANTIBODIES IN *PSEUDOMONAS* *AERUGINOSA* INFECTIONS

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(Received June 14, 1978)

Studying 347 sera of 49 patients suffering from *Pseudomonas aeruginosa* infection the mean titre value (MTV) calculated from the exponent of the binary logarithm of natural antibody (NA) titres, was found suitable to characterize the humoral immune status. As long as the organism is in equilibrium with the infection, the NA level rises. In septic shock, before death or at the development of a massive infection, the NA titre decreases rapidly. The decrease may be due partly to the permeability increasing effect of endotoxin and antigen-antibody reactions exerted on the capillaries. Consequently, in the most severe phase of sepsis, when bacteria enter the circulation less NA is available to fight against them. This might be a cause of the still very high lethality of septic shock due to Gram-negative bacteria. Finally, when applying the MTV one always has to consider that despite its advantages it gives less information than the Backhausz immunogram.

The immunogram representing the titre of certain natural antibodies (NA) is characteristic of the humoral immune reactivity [1, 2]. In our earlier studies the immunogram was found to change characteristically with the severity of the infection caused by *Pseudomonas aeruginosa* [3]. Accordingly, the NA level rose during the complications and at the peak of the infection, titre dispersion (simultaneous rise and decrease of different NA-s) could be observed, while in septic shock and before death the majority of the NA titres decreased by a velocity of 1.3–3.0-day halving time.

These observations were of qualitative character. The present study was aimed at the determination of the quantitative conditions. Observations were started before the infection and were continued during each stage of the complications due to *P. aeruginosa* until death or recovery of the patient. Values obtained in the different stages were compared to one another as well as to the NA level of healthy controls. (The Hungarian normal values being known [1, 9, 10], control values were required only for statistical analysis.)

Patients and methods

Patients. NA-s were determined parallel with the IgG, IgA and IgM level [4] in 287 sera of 17 male and 23 female patients of an acute respiratory ward, in 36 sera of 3 male and 6 female patients of a chronic ward and in 24 sera of 5 healthy persons. Distribution according to diagnosis and severity of the underlying disease and multiple bacterial complications (bronchitis, pneumonia, urinary tract infection, thrombophlebitis, injection abscess) of the patients

and the aetiology of the complications have been dealt with in detail earlier [4]. *Pseudomonas* infection was dominant in each patient. Mean age of the acute patients was 47.5, of the chronic patients 35.5 and of the controls 47.2 years.

Determination of NA-s. Blood sampling was repeated from acute patients at the onset of the illness every 2 to 3 day and later during convalescence at 6–7-day intervals, from chronic patients at 3-week intervals and in the controls every 4th or 5th day. The antibody titres to *Salmonella typhi* O, *Shigella sonnei*, *Shigella flexneri* 1a, 2b, 3, 6, *Escherichia coli* O26 and O55 were determined in each serum by passive haemagglutination. Haemolysis inhibition was used for estimating the staphylococcus α -antitoxin (S. α -a) level. For details of the techniques, see references [1, 5–8]. Only the titres of NA-s being frequent in the population were determined [1, 9, 10], so as to be able to evaluate its changes in each case. The anti-A and anti-B isoagglutinins and heterohaemagglutinins were not subjected to study, as part of the patients suffered from tetanus and was given tetanus anatoxin or antitoxin, in which group-material A is present and may influence the hetero-haemagglutinin titre [3].

When evaluating the S. α -a level the few sera obtained from patients with staphylococcal infection have not been taken into consideration. Owing to this and to the fact that the α -a value was expressed in international units, the changes of the S. α -a level were evaluated separately.

Mean titre. To express the quantitative changes in a simple way, the mathematical mean of the binary logarithm of 8 NA titres was used. Thus, a mean titre value (MTV) was obtained. As the same NA-s were studied and serial examinations were performed with each person, this calculated value gave reliable information on the changes of the immune status.

Grouping of sera. As in the case of immune globulin classes [4], sera were grouped according to the stage and severity of the complications. The following categories were applied: (1) free of complications; (2) onset of complications; (3) ongoing complications; (4) culmination of complications; (5) sepsis; (6) septic shock; (7) improvement; (8) convalescence; (9) blood sample on the day of death. According to severity: (A) free of complications; (B) mild; (C) moderately severe; (D) serious; (E) lethal. Accordingly, each blood sample is characterized by a number and a letter. E.g. 8/D indicates the serum of a patient in the stage of convalescence after severe complications.

Statistical analysis. The values for the individual groups were compared with Student's *t* test or with Wilcoxon's or Mann-Whitney's method.

Results

Healthy controls. The NA titres of the control sera tallied with the mean values obtained in other studies in Hungary [1, 9, 10]. For statistical evaluation, our own data were only used.

Acute patients. Changes of the MTV during complications in groups of different severity grade (Fig. 1). No significant differences were observed in cases with mild complications as compared to the controls, in full agreement with our previous studies when only slight changes of the NA levels could be detected in the case of mild complications [3]. In cases of moderate severity, the MTV rose rapidly from the beginning of complications until their culmination ($p < 0.01$) and decreased gradually during improvement and convalescence. This corresponds to the bell-shaped course of the individual NA titres [3]. In severe complications the already low NA level at the admission showed a further decrease at the onset of complications. During the complications the MTV rose and in the case of the frequently developing sepsis the rise became significant ($p < 0.05$). In septic shock a sudden and similarly significant decrease ($p < 0.05$) could be observed. In convalescence the MTV became normal.

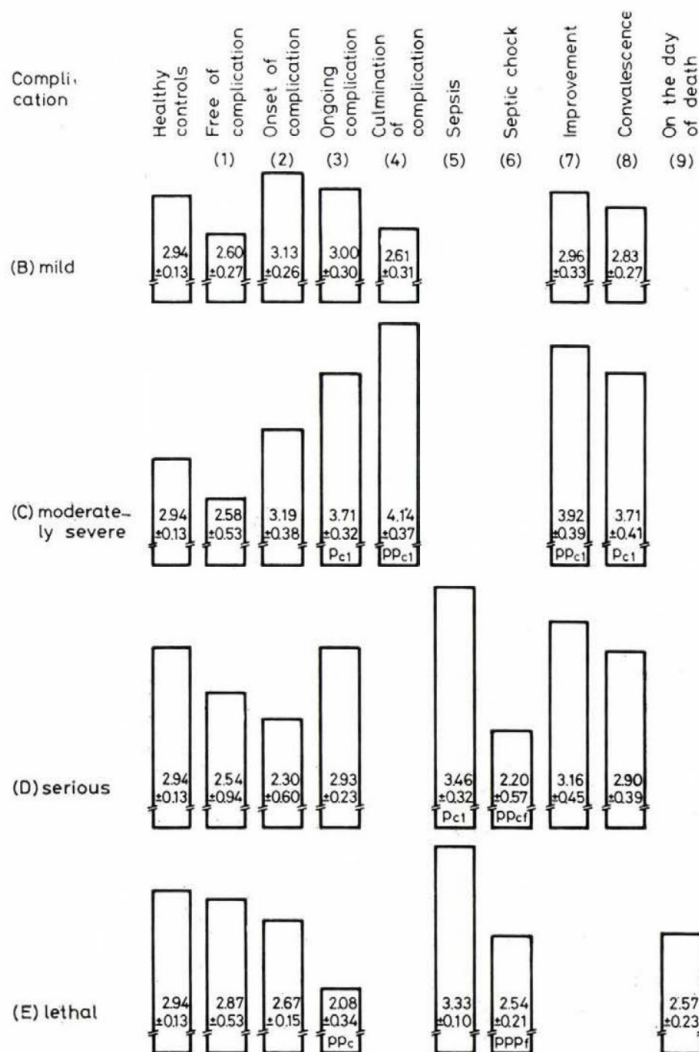


Fig. 1. Mathematical mean ($\pm$ standard error of the mean) of $^2\log$ exponent of 8 natural antibody titres in *P. aeruginosa* complications according to severity and stage. Acute patients. p_c = significant deviation as compared to controls; p_i = significant deviation as compared to group free of complication; p_l = significant deviation as compared to former group; p = < 0.05 ; pp = < 0.01 ; ppp = < 0.001 .

Lethal complications. The reduced MTV found at the beginning of complications decreased to a low value as compared to the controls during the complication ($p < 0.01$). In this group too, the MTV rose slightly over the normal value in sepsis, while in septic shock a sudden decrease occurred ($p < 0.001$). Death took place usually in the shock state. Values measured before death were not significantly lower than in other non-lethal septic shock states.

IgG and IgM type NA-s in different phases of complications. When evaluating the NA titre regarding all severity groups *in toto*, the MTV calculated from 8 NA-s showed characteristic changes in different stages of the complications. These changes showed a striking similarity with that of the IgM level in the same patient [4]. Therefore, the changes of the MTV were compared with those of NA-s belonging to the IgM (*S. flexneri* 3) and to the IgG class (*S. α-a*) [1]. As shown in Fig. 2 the changes of the IgM type *S. flexneri* 3 were found to correspond to the changes of both the MTV calculated from 8 NA-s and of the IgM level. Common characteristic was the low values of their levels in still complication-free patients, their gradual rise in the period of complications which resulted in significantly surpassing the normal level at the peak of the complications and in sepsis, as well as the sudden decrease both in shock and before death. In contrast the changes of the *S. α-a* titre resembled those of the IgG level [4]: values were low both at the onset and during the complications and did not surpass the level of the normal controls either at the culmination of the complication or in sepsis. Both in septic shock and before death the values decreased significantly.

Chronic patients. MTV of chronic patients surpassed the value of the controls in the complication-free period. During mild complications the MTV rose and became significantly higher than that of the controls ($p < 0.01$). During improvement and convalescence the values returned to the initial level (Fig. 3).

The antibody level of both *S. flexneri* 3 and *S. α-a* displayed the same characteristics.

Discussion

The origin of the NA-s has not yet been completely elucidated. In the opinion of certain authors their formation is genetically determined. According to others, endogenous and exogenous antigens induce their synthesis. Finally, a double origin has also been assumed [8, 11, 12].

The NA-s appear to react with every antigen except the own antigens found in the circulation [11, 13]. They play a role in immunological maturation [11, 14, 15], by recognizing foreign antigens [11, 16, 17]. They are indispensable in chemotaxis and phagocytosis [18, 19] and their presence contributes to the release of the immune response [20]. A relationship between the NA level and the endotoxin tolerance ensuring the survival of bacterial infections appears to be likely [21]. The bactericidal effect of the serum shows a linear relationship with the NA content [12] due to the phenomenon that as long as no antigen-specific antibody is present, the antigen attaches to the NA-s. Subsequently, they are bound to properdin and this complex inactivates (binds) the complement system [22-24]. The bactericidal effect of the serum

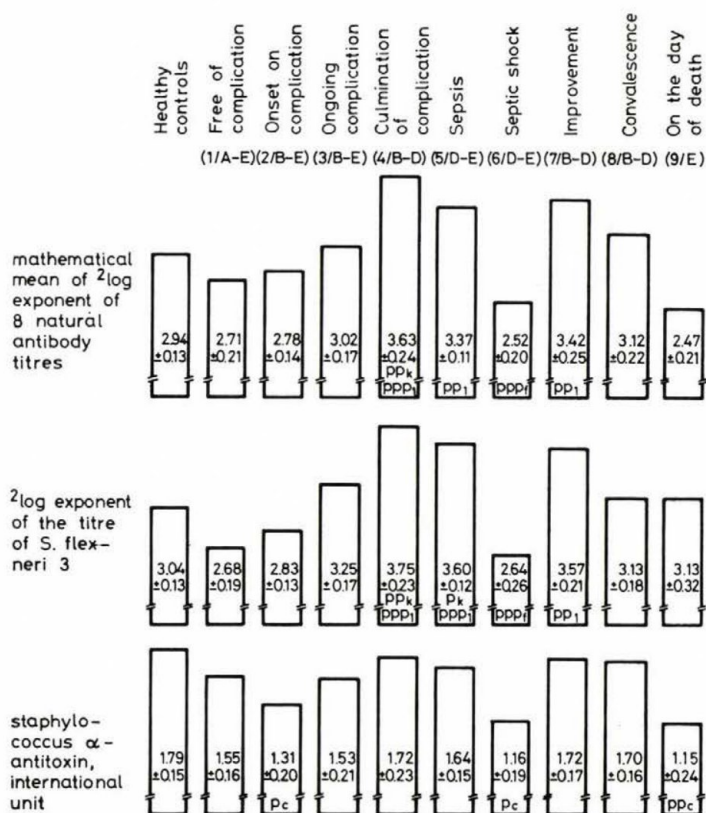


Fig. 2. Mathematical mean ($\pm$ standard error of the mean) of 2log exponent of 8 natural antibody titres, titre of *S. flexneri* 3 and level of staphylococcus α -antitoxin in different stages of *P. aeruginosa* complications. Acute patients. A = free of complication; B = mild complication; C = moderately severe complication; D = serious complication; E = lethal complication. Other symbols are identical with those of Fig. 1

depends on its complement content in the first place; bacteriolysis is potentiated by lysozyme and agglutination by conglutinins [25, 26].

This explains the proportional relationship between the humoral immune reactivity and the quantity of NA-s [1]. The lower the NA level the weaker the natural resistance and this results in an increased occurrence of different infections [2].

Our results are in good agreement with these facts. The high NA level during complications, at their culmination or during convalescence indicates that the humoral resistance is in equilibrium with the infection. The significant decrease of the MTV in septic shock and before death shows that the equilibrium has shifted towards the infection. The increase in MTV may be explained partly by the increased permeability of the anatomical barriers, resulting

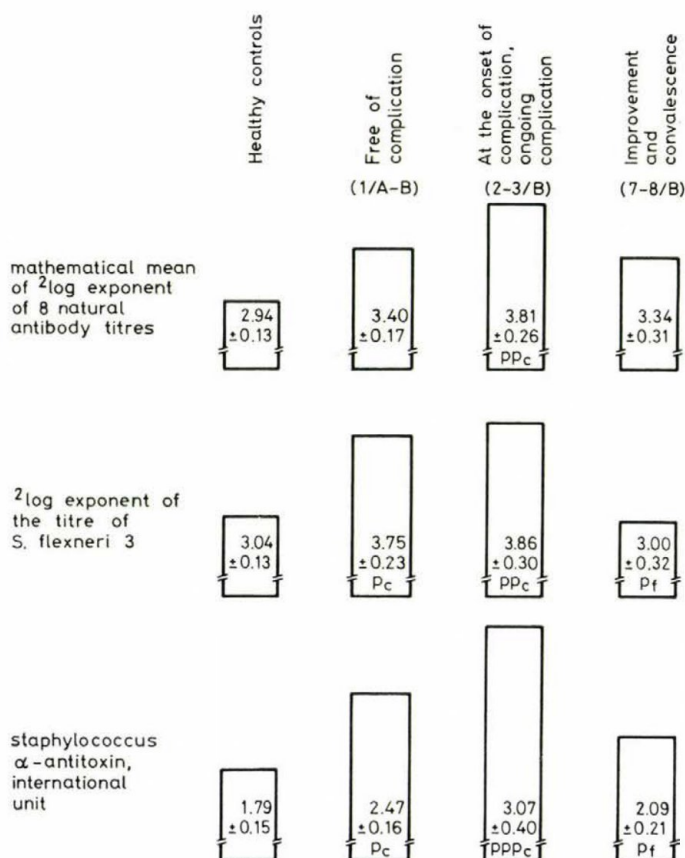


Fig. 3. Mathematical mean ($\pm$ standard error of the mean) of $2\log$ exponent of 8 natural antibody titres, titre of *S. flexneri* 3, and the level of staphylococcus α -antitoxin in different stages of *P. aeruginosa* complications. Chronic patients. Symbols identical with those of Fig. 1

in stronger antigen stimuli in infections, and partly by the endotoxins of Gram-negative bacteria, which are good stimulators of NA production [12].

The decrease of the circulating NA-s may be due to the formation of antigen-antibody complexes in a large quantity when the number of invading environmental antigens suddenly rises and the RES eliminates the developed complexes rapidly from the circulation [27]. The question is whether the antigen-antibody reactions by themselves provide a suitable explanation for the rapid, 1.5-3.0-day halving time decrease of the level.

The NA level diminishes rapidly in septic shock, at the onset of massive infections and before death, together with a sudden decrease of immune globulins and other serum proteins [4, 28]. During complications, the titre of the IgG type *S. α*-a changed similarly as the IgG class and the titre of the IgM type *S. flexneri* 3 similarly as the IgM class. The MTV changed parallel with the

IgM level, as the majority of the NA-s measured belonged to the IgM class [1]. The difference in behaviour of the IgG and IgM may be the consequence of the different diffusion coefficients of the two immune globulin types [4, 29]. All these suggest that not only the attachment of different antigens entering the organism may be responsible for the rapid decrease of NA-s, but the endotoxins and by-products of antigen-antibody reactions (histamine and kinins) also contribute to this effect by increasing the permeability of capillaries. In this case the different globulins leave the intravascular space at a rate corresponding to their diffusion coefficient. Consequently, at a critical period the mass of NA-s available for the fight against antigens (bacteria) invading the circulation diminishes and so the quantity of immune globulins transported to the site of action, the inflammatory area, also decreases. Considering the importance of NA-s in the defence against microorganisms, one may assume that the rapid and significant decrease of the level is one of the causes of the approximately 50% lethality of septic shock due to Gram-negative bacteria. The fatal outcome of septic cases may be prognosticated by the low NA level early after the onset of complications. The NA values of the survivors were namely significantly higher in the same period (Fig. 1, group 3/D: MTV 2.93; group 3/E: 2.08; $p < 0.05$). At this phase of the illness no other signs were found to refer to the later course of the complications. The IgM level showed similar changes in these patients [4]. Besides prognostic possibilities, these results may open new ways in the treatment of severe septic shock. The elevated NA level of chronic, complication-free patients raises further questions. Can this ensure their peaceful commensalism with the mass of facultative pathogenic Gram-negative bacteria [30]? Their IgM level does not seem to support this theory, on the other hand, large quantity of IgG was found in these patients [4]. The question arises whether the majority of their NA-s belonged to the IgG class.

Finally, it may be mentioned that the MTV, being a single number, is easier to use than the immunogram and gives a quick survey of the humoral immune status. At the same time it provides significantly less information than the immunogram, e.g. it does not indicate the type of NA-s and the immune globulin classes showing the most expressed changes.

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EFFECT OF KCN ON THE MOTILITY OF TREPONEMA PALLIDUM

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Virulent *Treponema pallidum* has been shown to survive in KCN-containing artificial medium. Oxygen uptake being sensitive to cyanide, the observation indicates that treponemes do not have a cytochrome oxidase system. KCN had a killing effect only at a 15–30 mM final concentration. The data show that the Budapest strain of *T. pallidum* is an anaerobic organism.

Studies of *Treponema pallidum* have substantiated the conclusion of its being an anaerobic organism [1]. Lately new information pertinent to the physiology of this microorganism has been obtained from studies on its survival *in vitro* [2–4] and from immunization experiments [5, 6]. Because the cultivation of *T. pallidum in vitro* was unsuccessful, aerobiosis was supposed [7] on the basis that *in vivo* the organism grows under the pO_2 of tissue [1]. In subsequent studies O_2 uptake by treponemes has been demonstrated and conclusions that O_2 consumption was linked to cytochrome oxidase activity are indicated by evidence that O_2 uptake was inhibited by cyanide [7]. Because it is impossible to produce treponeme suspension in the absence of tissue debris, we supposed that the treponemes would be killed by cyanide *in vitro*, too, if they would be really aerobic organisms.

In the present paper we describe *T. pallidum* survival in medium completed with KCN.

Materials and methods

The virulent Budapest strain of *T. pallidum* was used throughout [8]. The strain has been maintained in white New Zealand rabbits and was used to infect rabbit testicles which were harvested 7 days later to prepare the *T. pallidum* suspension [1]. X-ray irradiation (500 r) and 10 mg cortisone acetate daily permitted minimal antibody production. The cells found in the tissue extracts were removed by low speed centrifugation [3].

T. pallidum was extracted in a medium [9] containing 88 μ M tin chloride [2] and the extract was adjusted to approximately 4×10^7 *T. pallidum* per ml cell density in the same medium. The suspension was divided into 1 ml amounts and completed with KCN diluted in saline. The concentrations of KCN in the medium were 0.025, 0.05, 0.11, 0.23, 0.47, 0.95, 1.9, 3.8, 7.6, 15.3 and 30.7 mM. The tubes were kept under vacuum. Incubation was done in 10% CO_2 and 90% N_2 atmosphere at 35 °C and the specimens were examined by dark field microscopy at 24 hr intervals.

Results

After a 24 hr incubation, 100% motility was observed at 7.6 mM and higher dilutions of KCN. Even in the presence of 15–30 mM KCN the motility of *T. pallidum* was 12–32% at 48 hr (Fig. 1). Figure 2 shows an experiment in the presence of 1.9–30.7 mM KCN after a longer incubation period. No significant inhibition of treponeme motility was noted on increasing the KCN level to 15–30 mM (at this concentration the medium becomes hypertonic).

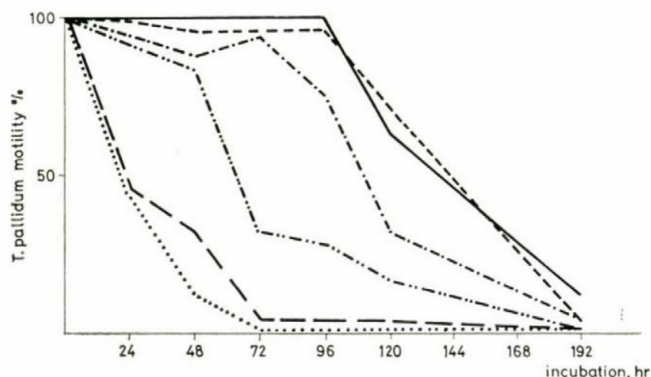


Fig. 1. Motility of *T. pallidum* in KCN-containing medium; KCN concentration, mM: - - - - - 1.9, - . - . - 3.8, - .. - .. - 7.6, - - - - - 15.3, 30.7, ——— control

Discussion

The motility of treponemes in the presence of KCN (Fig. 2) indicates that this energy source is independent of cytochrome oxidase.

The rate of oxygen uptake assumed as a function of treponemes [7] is, in our opinion, not associated with a functioning cytochrome system but is due to tissue debris in the suspension containing treponemes. We could accept

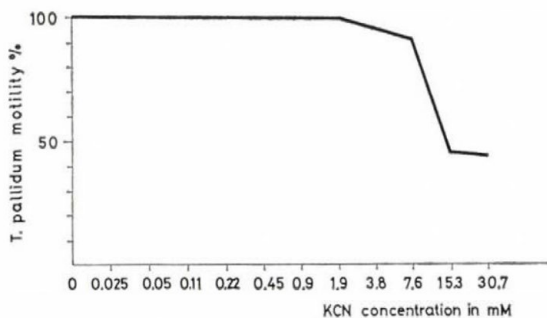


Fig. 2. Variation of *T. pallidum* motility in the presence of KCN after 24 hr incubation *in vitro*

that the cytochrome system was inhibited by cyanide on mitochondria of the suspension because both the treponemes and mitochondria have a similar sedimentation coefficient. Mitochondria inhibited by cyanide could not utilize oxygen. If the treponemes had a cytochrome system they would not be able to survive during 96 hr in a KCN-containing medium. The optimum temperature for O_2 uptake also contradicts to cytochrome being present in treponemes because at 38 °C they hardly survive in artificial medium and even under *in vivo* circumstances [10].

COX and BARBER [7] have shown that the O_2 uptake of *T. pallidum* is not inhibited using HOQNO or rotenon, whereas these substances completely inhibited the O_2 uptake by *Leptospira* B 16. Although oxygen consumption is inhibited by mitochondrial inhibitors in brain slice [11], COX and BARBER [7] have not written about the possibility of O_2 uptake by hostile mitochondria and cell debris in the treponeme suspension isolated from the orchitic testes. It may be assumed oxygen was consumed by tissue suspension as a *T. pallidum* suspension always contains cell debris and mitochondria [2]. If we accept the theory that the oxidized intermediates of treponemes are toxic, the toxic effect would be higher if *T. pallidum* was anaerobic, and the oxygen uptake in *T. pallidum* suspension would be inhibited by mitochondria too, because their reducing activity would be terminated.

The above data convinces us that virulent *T. pallidum* is a strict anaerobic organism. Because of their oxygen utilization, cell debris and mitochondria are very important for the survival of treponemes in helping them to maintain a reduced environment.

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CROSS-PROTECTION AGAINST SALMONELLA ENTERITIDIS INFECTION IN MICE*

I. IMMUNIZATION TRIALS

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Mice were immunized subcutaneously with live and killed vaccines, with and without complete adjuvant incorporating *Salmonella typhi-murium* M206, *Salmonella gallinarum* 9R, *Salmonella pullorum* Sp223 as well as homologous *Salmonella enteritidis* Se795. The animals were challenged 21 days post-vaccination with 100 LD₅₀ of virulent *S. enteritidis* 5694 SMR subcutaneously along with unvaccinated control mice. To assess the immunity against acute and chronic infections, the percentage of absolute survivors i.e. survivors without lesions and without the challenge organism, was taken as the criterion. Live vaccines proved better than killed vaccines. Live vaccines with complete adjuvant induced a good protection. Cross-protection could be induced with the live vaccine with complete adjuvant against *S. enteritidis* infection in mice.

Mouse typhoid has been studied extensively as an animal model of human typhoid fever. Several vaccines, especially cross-protecting vaccines, have been applied [1-9], and live vaccines were found superior to killed vaccines [8, 10, 11]. Live vaccine with Freund's complete adjuvant was found to be superior against fowl typhoid [12]. The present paper reports on results of cross-protection induced by live and killed vaccines with or without complete adjuvant incorporating rough, avirulent and less virulent strains antigenically heterologous to *S. enteritidis*.

Materials and methods

Mice. Swiss male mice, six to eight weeks of age, 16 to 20 g in weight, supplied by the Division of Biological Products, Indian Veterinary Research Institute, Izatnagar, were used. Vaccinated mice of each group were housed and fed separately.

Organisms. *S. typhi-murium* M206 (1,4,5,12:i:1,2); less virulent strain, obtained from the University of Adelaide, Australia, through the kind courtesy of Dr. D. Rowley. Vaccine dose: 5×10^5 colony forming units (c.f.u.) per 0.2 ml.

S. gallinarum 9R; a rough strain evolved by Dr. H. W. SMITH, obtained from the Central Veterinary Laboratory, Weybridge, by the Enterobacteriaceae Laboratory, I.V.R.I. Izatnagar. Vaccine dose: 10×10^7 c.f.u./0.2 ml.

S. pullorum Sp223 (1,9,12:-:-); an avirulent strain kindly supplied by Dr. F. M. COLLINS, Trudeau Institute, Saranac Lake, New York. Vaccine dose: 10×10^7 c.f.u./0.2 ml.

S. enteritidis Se795 (1,9,12:gm:-); a less virulent strain kindly supplied by Dr. F. M. COLLINS. Vaccine dose: 10×10^6 c.f.u./0.2 ml.

S. enteritidis 5694 SMR (1,9,12:gm:-); virulent strain resistant to streptomycin (30 µg/ml), used as the challenge strain. Kindly supplied by Dr. F. M. COLLINS. LD₅₀ by the subcuta-

* Part of Ph. D. thesis submitted by the senior author to Agra University, Agra, India.

neous route was $10^{5.4191}$ c.f.u. The organism was maintained by medium-mouse-medium passage.

Complete adjuvant. Arlacel A (Atlas Chemical Industries, U.S.A.) 1.5 ml; liquid paraffin (H.P.C.) 8.5 ml. The two ingredients were mixed in a Waring blender and sterilized by autoclaving at 121 °C for 30 min. To this was added sterile trypsinized extract of *Mycobacterium phlei* (kindly supplied by Dr. B. R. GUPTA, I.V.R.I. Izatnagar) at the rate of 4 mg/ml.

Vaccines. Base preparation. After verifying the purity and serological identity of an 18 hr broth culture, the preparation was adjusted to double the final dose.

Killed vaccines. To the base preparation an equal quantity of sterile normal saline solution (NSS) was added and the organisms were heat-killed in a water bath of 60 °C for one hr.

Killed vaccines with complete adjuvant. To the base preparation heat-killed at 60 °C for one hr, an equal amount of sterile complete adjuvant was added and the mixture blended with a sterile syringe fitted with 16G hypodermic needle.

Live vaccines. To the base preparation an equal amount of sterile NSS was added.

Live vaccines with complete adjuvant. To the base preparation, an equal amount of sterile complete adjuvant was added and the mixture blended thoroughly with a sterile syringe fitted with a 16G needle.

Route of vaccination. Subcutaneous. Dose 0.2 ml.

Challenge. Twenty one days after vaccination all the surviving mice along with a batch of unvaccinated control mice (of the same lot and maintained separately) were challenged subcutaneously with 100 LD₅₀ dose of *S. enteritidis* 5694 SMR. The mice were observed for a period of 28 days post-challenge. Animals dead during the observation period were examined for the presence of any lesion and attempts were made to isolate the challenge organism on a streptomycin (30 µg/ml), MacConkey agar plate. After 28 days all the surviving mice were sacrificed and examined for lesions and challenge organism. Only those mice which survived the observation period without any lesion or challenge organism were considered absolute survivors.

Statistical analysis. The data were analysed statistically by the Chi square test with Yate's correction.

Results

Table I presents the results of the immunization trial. Killed vaccines, either homologous or heterologous, failed to induce significant protection. Killed vaccines with complete adjuvant were slightly better but also failed to induce significant immunity except *S. pullorum* Sp223 killed vaccine with complete adjuvant which induced a marginally significant protection.

Among the live vaccines, vaccines with *S. typhi-murium* M206 and *S. pullorum* Sp223 as the immunizing agents failed to induce significant protection. The rough *S. gallinarum* 9R live vaccine afforded significant protection though of lesser degree than the homologous *S. enteritidis* Se795 live vaccine.

Live vaccines with complete adjuvant induced a very good protection. Heterologous vaccines with complete adjuvant induced very good protection closely comparable to that afforded by the homologous live vaccine.

Discussion

The subcutaneous route of immunization and challenge has been selected as the intraperitoneal [4, 13] and the intravenous routes [14, 15] have been criticized. Intraperitoneal injection might result in an unwanted local effect

Table I

Immunization trial against S. enteritidis 5694 SMR infection in mice

Vaccine	No. of mice				Percent of absolute for survivors
	Vaccinated	Survived challenge after 21 days of vaccination	Survived challenge up to 28 days post-challenge	Free from challenge organism	
<i>S. typhi-murium</i> M206					
Killed	15	13	1	0	0
Killed with adjuvant	15	13	4	4	30.77
Live	15	13	4	4	30.77
Live with adjuvant	13	8	6	6	75.00***
<i>S. enteritidis</i> Se795					
Killed	15	14	4	3	21.43
Killed with adjuvant	14	11	4	4	36.36
Live	15	12	11	11	91.66***
Live with adjuvant	14	6	6	6	100.00***
<i>S. gallinarum</i> 9R					
Killed	14	11	2	2	18.18
Killed with adjuvant	14	12	3	3	25.0
Live	15	14	8	8	57.14**
Live with adjuvant	15	15	9	9	60.00**
<i>S. pullorum</i> Sp223					
Killed	14	10	2	2	20.00
Killed with adjuvant	13	9	4	4	44.44*
Live	15	13	5	5	38.46*
Live with adjuvant	13	8	7	7	87.50***
None (Control)	—	14	1	0	0.00

* χ^2 test significant at 5% level** χ^2 test significant at 1% level*** χ^2 test significant at 0.1% level

and the intravenous route bypasses the lymphatic barriers. There are counter-claims on the use of these routes. Natural infection being through the oral route, the best method for any study should be the same route, but this involves difficulties and the large inoculum may counteract the acid reaction of the stomach. Therefore, we have selected the subcutaneous route, as the second best.

Though GREENWOOD *et al.* [16] and SCHUTZE [6] reported the effectiveness of killed vaccines, they used mean survival time as the criterion. The inefficiency of killed vaccines recorded in the present investigation has already

been reported [8, 10, 11]. In the present investigation the percentage of absolute survivors was the criterion as this would indicate the survivors of both acute and chronic infections including the carriers. Since these are facultative intracellular parasites, the real efficacy of a vaccine would be indicated only when the absolute survivors are taken into consideration.

COLLINS [3, 17] claimed protection with killed vaccines incorporated in Freund's complete adjuvant against a lethal challenge with *S. enteritidis* 5694 SMR. The apparent discrepancy between these results and those of the present study might be due to difference in the dose schedule and perhaps to a minor extent to the adjuvants used.

It is of importance that a rough *S. gallinarum* 9R live vaccine could induce cross-protection against heterologous challenge with *S. enteritidis* 5694 SMR.

Adjuvants have already been used with live vaccines in animals. VALLEE and RINJARD [18] used adjuvant along with *Mycobacterium paratuberculosis* as the immunizing agent against Johne's disease. Freund's complete adjuvant has been used with *S. gallinarum* 9R against fowl typhoid [12].

In the present study live vaccines with complete adjuvant have proved highly useful in affording cross-protection. *S. gallinarum* 9R incorporated in complete adjuvant induced an even better protection. There is, however, a possibility of the enhancement of virulence as could be seen in the case of smooth strains (Table I) resulting in post-vaccinal mortality. The observation of MACKANESS [19] that adjuvants might enhance the virulence, proved correct.

It would, however, be safe to use complete adjuvants with rough strains to tap the nonspecific expression of immunity.

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CROSS-PROTECTION AGAINST SALMONELLA ENTERITIDIS INFECTION IN MICE*

II. HUMORAL IMMUNE RESPONSE

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Mice were immunized subcutaneously with live vaccines and live vaccines with complete adjuvant incorporating *Salmonella enteritidis* Se 795, *Salmonella typhi-murium* M206, *Salmonella gallinarum* 9R or *Salmonella pullorum* Sp223. They were challenged along with unvaccinated controls with 100 LD₅₀ of virulent *S. enteritidis* 5694 SMR subcutaneously on the 21st day post-vaccination. The humoral immune response was studied by assessing the sequential level of agglutinins, complete and incomplete somatic antibodies, opsonophagocytic antibodies, cytophilic antibodies and bactericidal antibodies before and after challenge. The level of these antibodies and the protection afforded by the particular vaccine is correlated and the possible involvement of a humoral mechanism is discussed.

There are few better examples of a scientific controversy than the immune response to mouse typhoid which still remains unsettled. The two Japanese schools headed by USHIBA and MITSUHASHI [1—6] were emphatic about the role of a cell-mediated immune mechanism in the protection against mouse-typhoid. The Australian workers headed by JENKIN and ROWLEY [7—19] were equally emphatic about the role of humoral factors to the complete exclusion of cell-mediated factors. These two rival schools of thought have been getting support from workers all over [20—27]. The present paper records the role of humoral factors in protection especially cross-protection against *Salmonella enteritidis* infection in mice and the next paper will deal with the role of hypersensitivity reactions in the immune response against this infection.

Materials and methods

The animals, organisms and vaccines used, and the technique of challenge have already been described [28].

Collection of sera for study of humoral factors. Sera from a randomly selected group of four mice were obtained by collecting the blood from the infra-orbital plexus and pooling the serum (equal quantities). The blood samples were collected on the day of vaccination (0 day), the 10th and 21st day post-vaccination, and on 7th, 14th, 21st and 28th day post-challenge. The pooled samples were distributed in six tubes and stored at -20 °C until used. All the tests were conducted using a single batch of reagents and the serum samples collected from a group of mice immunized with a particular vaccine and challenged were tested together to avoid

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variation. All serum samples were inactivated at 56 °C for 30 min to remove non-specific factors.

Detection of agglutinins by tube agglutination test. Heat-killed (60 °C for one hour) suspension of *S. enteritidis* 5694 SMR, with its opacity adjusted to No. 3 of Brown's scale, was used as antigen. Twofold dilutions of the serum samples in 0.5 ml amount were carried out in 10 × 75 mm Corning test tubes and to this 0.5 ml of antigen was added and incubated at 37 °C for one hour and at 4 °C overnight. Agglutination was measured by the settling pattern, clearness of the supernatant and the nature of sedimented particles. The titre represented the reciprocal of the highest dilution of the serum showing 50% agglutination.

Detection of complete antibodies by the indirect haemagglutination test. The methods adopted by INGRAM and MALCOMSON [29] were followed. Sheep erythrocytes (SRBC) were sensitized with saline extract of *S. enteritidis* 5694 SMR. To a 1% suspension of freshly collected washed SRBC an equal volume of antigen was added. The mixture was incubated at 37 °C for one hour. The sensitized SRBC were then washed three times with normal saline solution (NSS) by centrifugation and reconstituted to 1%.

Heat-inactivated serum samples were absorbed with 10% SRBC at 4 °C overnight to remove heterophile agglutinins. To serial twofold dilutions of the 0.2 ml samples, 0.2 ml of sensitized SRBC was added and incubated in a water bath at 37 °C for 2 hr. The reciprocal of the highest dilution of the serum giving 50% haemagglutination was considered the end titre.

Detection of incomplete antibodies by antiglobulin haemagglutination test. The method adopted by INGRAM and MALCOMSON [29] was followed with modifications.

Anti-mouse globulin was prepared in rabbits as follows. Mice were sacrificed by bleeding from the infra-orbital plexus and the sera were pooled. The globulin was separated with ammonium sulphate according to FUDENBERG [30]. Rabbits were given daily injections of the globulin in 0.5, 0.5, 1.0, 1.5, 2.0 and 2.0 ml doses intravenously. Seven days after the last injection, blood was collected by heart puncture from the rabbit, and the serum separated formed the anti-globulin. The anti-globulin was heat-inactivated at 56 °C for 30 min and absorbed with 50% SRBC at 4 °C overnight. It was titrated using a known positive serum in a suitable dilution.

The tubes showing no haemagglutination in the indirect haemagglutination test were removed and the sensitized SRBC were washed three times with NSS by centrifugation and reconstituted to 0.2 ml. To these tubes 0.2 ml of rabbit anti-mouse globulin was added and incubated in a water bath at 37 °C for 2 hr. The reciprocal of the highest serum dilution showing 50% haemagglutination was taken as the end-titre.

Detection of opsono-phagocytic antibodies. Five healthy mice were sacrificed and the spleens were collected aseptically in cold Hank's BSS. After rinsing, the spleens were teased against a sterile nylon mesh to disperse the spleen cells. Coarse particles were removed by centrifugation at 500 rpm in a Remi T8 centrifuge in polythene centrifuge tubes. The supernatant was used to form a monolayer of spleen cells. Approximately 10⁸ spleen cells were added to each cavity of acid and alkali treated cavity slides. The monolayer was allowed to be formed by incubation at 37 °C for one hour.

An 18-hour-old nutrient broth culture of *S. enteritidis* 5694 SMR was checked for purity and washed three times with NSS by centrifugation. The final pellet was diluted so as to contain 1000 bacteria per ml. Equal amounts of one in five diluted heat inactivated test serum samples and the bacteria were allowed to interact for 30 min at 37 °C. Subsequently, the bacteria-serum mixture was centrifuged at 4000 rpm in a Remi T23 centrifuge for 15 min and the bacteria were washed three times with sterile NSS to remove the excess antibody. The bacteria were reconstituted to the original volume. A control of normal serum was also included.

To each slide containing a spleen cell monolayer, 0.2 ml of sensitized bacteria in Hanks' BSS was added and allowed to interact at 37 °C for 30 min. The reaction was suspended by washing the monolayer with cold Hanks' BSS at least four times. The slides were fixed with methanol and stained by Leishman stain.

Percentage of phagocytosis was estimated by examining 200 cells for each slide (i.e. for each serum sample) under the microscope. The number of spleen with one or more bacteria inside per hundred spleen cells counted, constituted the percentage of phagocytosis.

The opsonic index was calculated using the formula,

$$\text{Opsonic index} = \frac{\text{Percentage of phagocytosis of test serum treated bacteria}}{\text{Percentage of phagocytosis of normal serum treated bacteria}}$$

Detection of cytophilic antibodies. The techniques used by TIZARD [31] for the detection of cytophilic antibodies were followed. Spleen cell monolayers were prepared as described above.

Sensitization of the monolayer with serum was performed as follows, samples heat-inactivated at 56 °C for 30 min were diluted twofold from 1:5 to 1:160 in Hanks' BSS in each cavity of spleen cell monolayer was replaced with the serum sample. Normal mice serum at 1:5 dilution formed the control. The serum was allowed to sensitize the spleen cells at 37 °C for 30 min. Later on, the slides were well washed with Hanks' BSS at least three times to remove excess serum.

To the sensitized monolayers, the unsensitized *S. enteritidis* 5694 SMR in the log phase diluted in Hanks' BSS so as to contain 1000 organisms/ml was added and incubated at 37 °C for 30 min. The reaction was suspended after 30 min by washing the monolayers with cold Hanks' BSS four times. The slides were fixed in methanol, stained with Leishman and examined under oil-immersion. Spleen cells with at least two bacteria attached to its surface were taken as positive. The reciprocal of the highest dilution of serum which sensitized the monolayer so that at least 50% of the monolayer cells present two or more bacteria adhered to the spleen cell, formed the end-titre.

Detection of bactericidal antibodies. The procedure described by MITTAL and INGRAM [32] has been followed.

For complement, 15 guinea pigs weighing 500 g each were bled by heart puncture and the blood collected aseptically was allowed to clot. The clot was disturbed after two hours under sterile precautions and the serum was collected by centrifugation after keeping the suspension at 4 °C for 6 hr. The pooled serum from 15 guinea pigs was then redistributed in 2 ml aliquots in prechilled tubes. The complement was absorbed with the heat-killed culture of *S. enteritidis* 5694 SMR (at the rate of 0.3 ml of packed volume per ml of complement) at 4 °C for one hour. The bacteria were removed by centrifugation in cold and stored at -20 °C. Complement once removed was used on the same day and the balance was discarded. Complement was stored in the deep freeze for a maximum period of 60 hr.

A six-hour nutrient broth culture of bacteria was centrifuged at 4000 rpm in a Remi T23 centrifuge for 15 min and washed three times with sterile NSS by centrifugation. The final pellet was reconstituted in bactericidal diluent [33] so that one ml contained approximately 2000 to 3000 bacteria.

Serum samples were diluted twofold in 0.2 ml bactericidal diluent starting from neat, 1:5, 1:10, etc. To this, absorbed complement was added undiluted in 0.1 ml amounts and 0.2 ml aliquots of bacterial suspension were added and incubated at 37 °C for one hour. Controls consisted of serum samples plus bacteria without complement as well as complement plus bacteria without serum sample. After one hour one loopful (0.03 ml) was plated over well-dried MacCONKEY's agar plates in duplicate for each dilution. The plates were incubated overnight and the number of colony forming units (c.f.u.) were counted. The results were graded as follows: ++++ no growth, complete bactericidal effect; +++ 25% growth as compared to serum control; ++ 50% growth, 50% bactericidal effect; + = 75% growth; - no difference between test and control plates.

The end-titre was taken as the reciprocal of the highest serum dilution able to exert a ++ bactericidal effect.

Results

Agglutinins. The results are presented in Table I. The live vaccines with complete adjuvant incorporating the smooth organisms viz. *S. enteritidis* Se 795, *S. typhi-murium* M206 and *S. pullorum* Sp 223 have induced a better response than their corresponding live vaccines. In all these groups there was a definite fall in the titre of agglutinins by the seventh day post-challenge and a recovery by the 4th day post-challenge. The rough vaccines, viz. *S. gallinarum* 9R live vaccine with and without complete adjuvant did not induce an agglutinin response even 21 days after vaccination.

Complete and incomplete antibodies. The results are represented in Table II. Even the rough strains are able to induce production of complete and incomplete antibodies. The level of these antibodies rises gradually up to 21 days post-challenge and then falls by 28 days post-challenge.

Table I

Agglutinin response in mice vaccinated with different vaccines and challenged with S. enteritidis 5694 SMR

Vaccine	Days post-vaccination			Days post-challenge			
	0	10	21	7	14	21	28
<i>S. typhi-murium</i> M206 live vaccine (M206 L)	0	20	80	0	10	80	80
<i>S. typhi-murium</i> M206 live vaccine with adjuvant (M206 LA)	0	40	160	20	160	640	80
<i>S. enteritidis</i> Se795 live vaccine (Se795 L)	0	80	320	20	80	1280	160
<i>S. enteritidis</i> Se795 live vaccine with adjuvant (Se795 LA)	0	160	320	40	320	2560	640
<i>S. gallinarum</i> 9R live vaccine (9R L)	0	0	0	40	160	1280	160
<i>S. gallinarum</i> 9R live vaccine with adjuvant (9R LA)	0	0	0	80	320	2560	640
<i>S. pullorum</i> Sp223 live vaccine (Sp223 L)	0	40	40	10	40	160	40
<i>S. pullorum</i> Sp223 live vaccine with adjuvant (Sp223 LA)	0	80	320	40	40	320	80
None (control)	0	ND	0	160	2560	NM	NM

Titres represent the reciprocal of the highest dilution of pooled serum showing 50% agglutination

ND = not done, NM = no mice survived, 0 = < 10

Opsono-phagocytic antibodies. The results are presented in Table III. The best response is seen in mice receiving homologous vaccines. The response showed an increase by the 7th day post-challenge and continued to rise. Figure 1 represents a spleen cell which has ingested *S. enteritidis* opsonized by the test serum sample.



Fig. 1. Opsono-phagocytic antibody activity against *S. enteritidis* 5694 SMR. $\times 1750$

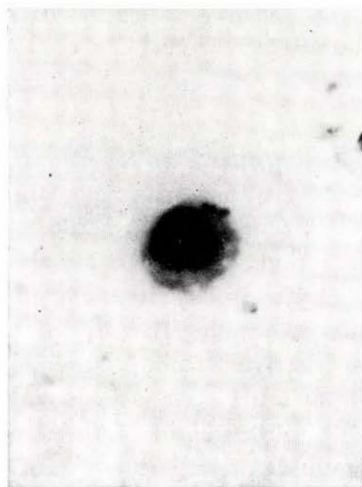


Fig. 2. Cytophilic antibody activity against *S. enteritidis* 5694 SMR. $\times 1750$

Table II

Complete and incomplete somatic antibody response (as detected by IHA and AGHA tests) in mice against S. enteritidis 5694 SMR

Vaccines		Days post-vaccination			Days post-challenge			
		0	10	21	7	14	21	28
M206 L	IHA	5	80	640	10240	20480	20480	5120
	AGHA	10	320	2560	20480	40960	40960	20480
M206 LA	IHA	5	640	2560	5120	5120	5120	2560
	AGHA	10	1280	5120	20480	40960	20480	10240
Se795 L	IHA	5	2560	5120	10240	40960	10240	10240
	AGHA	10	10240	20480	81920	81920	40960	40960
Se795 LA	IHA	5	2560	10240	1280	2560	10240	10240
	AGHA	10	20480	40960	20480	40960	40960	20480
9RL	IHA	5	160	640	2560	10240	20480	640
	AGHA	10	320	2560	10240	40960	40960	10240
9RLA	IHA	5	320	2560	2560	2560	5120	320
	AGHA	10	1280	20480	20480	40960	20480	2560
Sp223 L	IHA	5	320	640	640	2560	2560	5120
	AGHA	10	1280	2560	2560	20480	10240	20480
Sp223 LA	IHA	5	1280	1280	1280	40960	10240	20
	AGHA	10	10240	10240	20480	40960	40960	640
None	IHA	5	ND	20	5120	20480	NM	NM
	AGHA	10	ND	40	81920	81920	NM	NM

The titres represent the reciprocals of the highest dilution of serum showing the end points

ND = not done, NM = no mice survived

Table III

Opsonic index of pooled sera samples of mice against S. enteritidis 5694 SMR

Vaccine	Days post-vaccination			Days post-challenge			
	0	10	21	7	14	21	28
M206 L	1.0	1.6	1.33	2.0	3.0	5.0	6.33
M206 LA	1.0	1.66	1.66	2.66	4.0	9.33	10.00
Se795 L	1.0	1.0	1.0	2.33	4.0	6.66	8.66
Se795 LA	1.0	2.0	3.0	4.0	7.0	12.0	16.0
9RL	1.0	1.0	1.0	2.0	2.66	3.66	4.0
9RLA	1.0	1.0	2.0	5.0	6.0	6.0	7.0
Sp223 L	1.0	1.0	2.66	3.66	5.33	6.66	7.0
Sp223 LA	1.0	1.66	2.66	3.66	6.00	7.0	8.0
None	1.0	ND	1.0	2.0	4.0	NM	NM

ND = not done, NM = no mice survived

Cytophilic antibodies. The results are presented in Table IV. Except the homologous vaccines, none of the heterologous vaccines could induce production of cytophilic antibodies until the 21st day post-vaccination. The post-challenge response was also poor in mice vaccinated with rough vaccine. Figure 2 represents a spleen cell sensitized with serum showing bacteria adhering to its surface.

Bactericidal antibodies. The results are shown in Table V. Very low levels of bactericidal activity were observed against the virulent *S. enteritidis* 5694

Table IV

Cytophilic antibody response in mice against S. enteritidis 5694 SMR

Vaccine	Days post-vaccination			Days post-challenge			
	0	10	21	7	14	21	28
M206 L	0	0	0	0	5	10	20
M206 LA	0	0	0	0	5	20	20
Se795 L	0	0	0	5	10	20	40
Se795 LA	0	0	5	5	10	40	80
9RL	0	0	0	0	5	5	5
9RLA	0	0	0	0	5	10	5
Sp223 L	0	0	0	0	5	10	10
Sp223 LA	0	0	0	0	5	10	10
None	0	ND	0	0	10	NM	NM

0 = < 5, ND = not done, NM = no mice survived

The titres represent the reciprocal of the highest dilution of serum able to cause rosette formation with at least two bacteria, in at least 50% of the spleen cells

Table V

Bactericidal antibody response in mice against S. enteritidis 5694 SMR

Vaccine	Days post-vaccination			Days post-challenge			
	0	10	21	7	14	21	28
M206 L	0	0	5	0	5	1	0
M206 LA	0	1	10	1	1	5	0
Se795 L	0	0	5	0	5	5	0
Se795 LA	0	5	20	5	5	10	0
9RL	0	0	0	0	5	5	0
9RLA	0	0	0	0	1	1	0
Sp223 L	0	0	1	1	1	5	0
Sp223 LA	0	0	10	1	1	5	0
None	0	ND	0	1	10	NM	NM

ND = not done, NM = no mice survived

A titre of 1 represents neat serum

SMR. Live vaccines with complete adjuvant incorporating smooth strains induced a better response. Like with the agglutinins, there was a fall in titre by the 7th day post-challenge.

Immunization trials with these vaccines have been described in a previous paper [28].

Discussion

A significant result of the study was the fall in titre by the 7th day post-challenge in the sera of mice vaccinated with smooth strains, suggesting its possible involvement during the early challenge period. This involvement might perhaps be responsible for the clearance of the challenge organism. The absence of involvement of agglutinins in mice vaccinated with rough strains and the significant protection given by *S. gallinarum* 9R live vaccine with complete adjuvant [28] makes the role of agglutinins questionable. ORNELLAS *et al.* [27] also demonstrated agglutinins but could not emphatically confirm that they are responsible for protection. KENNY and HERZBERG [24, 25] emphasized on the bactericidal antibody which appeared much earlier.

The complete and incomplete somatic antibodies detected by indirect haemagglutination test and anti-globulin haemagglutination test, did not reveal any correlation with the level of protection. The study failed to confirm the assumption of BUXTON and DAVIES [34] that these antibodies are indicative of disease rather than immunity. The only significant feature is that the antibodies induced by the rough *S. gallinarum* 9R vaccines have been detected by saline extract of smooth *S. enteritidis* 5694 SMR coated onto sheep erythrocytes. This may be explained in two ways. The reaction may be due to the "O" antibodies induced by some "O" determinants present on the rough organism, or conversely the "R" antibodies induced by the "R" determinants have been detected by the few "R" determinants that may be present on the smooth *S. enteritidis* 5694 SMR and which coated the SRBC. The problem needs further study.

The response of the opsono-phagocytic antibody is best in mice vaccinated with the homologous live vaccine with complete adjuvant, followed by homologous live vaccine. Like agglutinins, no opsono-phagocytic antibody could be detected in mice after receiving the rough vaccines. *S. gallinarum* 9R live vaccine with complete adjuvant induced, however, a significant protection [28]. The level of antibody kept on rising even after challenge. In the face of a challenge, if the level of the opsono-phagocytic antibody rises, the host may respond by enlarging its defence architecture. TURNER *et al.* [19] have also recorded that opsono-phagocytic antibody appears by 5th day post-infection and reaches its peak by the 14th day. In the present study the peak level occurred on the 28th day post-challenge.

The single inoculation response of TIZARD [31] with sheep erythrocytes in mice could be noticed only in the case of *S. enteritidis* Se795 live vaccine with complete adjuvant which has induced cytophilic antibodies by the 21st day post-vaccination. All the other vaccines failed to induce cytophilic antibodies during the post-vaccination period and only during the post-challenge period could a mild response be detected. The response was the poorest with the rough strains; this confirms the results of USHIBA [1] and MITTAL [33]. ROWLEY *et al.* [18] studied the fixed cytophilic antibody around macrophages and not the level of free cytophilic antibodies in the serum. The contention that the cell-bound antibodies of peritoneal macrophages [18] are responsible for the protection against mouse typhoid can perhaps be agreed for intraperitoneal challenge. By the subcutaneous and intramuscular routes, the challenge organism faces the lymphatic barrier, and by the oral route, the enteric barrier. The claim that cell-bound antibodies are responsible for the protection, fails when a rough strain which cannot provoke the formation of cytophilic antibodies, is used as the immunizing agent.

For bactericidal assay, it is imperative that the virulent strain should be used as the test organism to stimulate natural conditions. It would serve no purpose to apply avirulent strains as has been done by ROWLEY [15] and DAVIES and KOTLARSKI [22]. KENNY [23] following the findings of ADLER [35] used smooth strains coated with extracts from the rough strain to demonstrate bactericidal antibody. This again would serve only to demonstrate a property on an entirely new configuration. The results obtained by KENNY [23] may not relate to protection. The titre of the bactericidal antibody in this investigation varies with the vaccine, but in general the response is poor. This may perhaps be due to the fact that virulent strain was used as test organism. There is a mild involvement of bactericidal antibody during the early post-challenge period like agglutinins in mice vaccinated with smooth strains.

Thus, the role of humoral antibodies in protection against *S. enteritidis* infection in mice is only marginal. Agglutinins and bactericidal antibodies show the involvement, but it is not essential. The complete and incomplete somatic antibodies detected by IHA and AGHA tests failed to correlate with protection. In the absence of cytophilic antibodies, rough strains induced good protection. Opsono-phagocytic antibodies kept on rising even after challenge, suggesting a minor role in strengthening the body defence.

It is therefore concluded that humoral factors are not essential for protection against *S. enteritidis* infection in mice. If present, they may, however, play a useful role.

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CROSS-PROTECTION AGAINST SALMONELLA ENTERITIDIS INFECTION IN MICE*

III. DELAYED HYPERSENSITIVITY REACTION AND CLEARANCE OF THE CHALLENGE ORGANISM

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Mice were immunized with live vaccines and with live vaccines with complete adjuvant incorporating *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella gallinarum* or *Salmonella pullorum*. On the 21st day after vaccination, the hypersensitivity reactions elicited by the mice to extracts of the challenge organism (*S. enteritidis* 5694 SMR) were assessed. The degree of delayed hypersensitivity reaction was compared with the level of protection induced by the vaccine. The role in protection of delayed hypersensitivity is discussed. Clearance of the challenge organism from the liver of previously vaccinated and unvaccinated mice was assessed quantitatively.

The results of immunization of mice with different vaccines against *Salmonella enteritidis* infection and the role of humoral factors in protection have been described [1, 2]. The present paper records the results of hypersensitivity reactions and the clearance of the challenge organism from the liver of vaccinated and unvaccinated mice. The role of delayed hypersensitivity as an attribute of cell-mediated immune response has amply been studied [3–9]. Clearance of the challenge strain had been the sole criterion of protection by many workers [3, 10–14].

Materials and methods

The mice, organisms and vaccines used and the technique of vaccination and challenge have been described previously [1].

Preparation of eliciting antigens. Saline extract. An eighteen hour broth culture of *S. enteritidis* 5694 SMR was inoculated into Roux flasks with nutrient agar slant and incubated at 37 °C. After 24 hr, the growth was washed with normal saline solution (NSS), purity was checked, and the culture was washed with NSS at least three times by centrifugation at 3000 rpm for 10 min each in a Remi T23 centrifuge. Opacity was adjusted to No. 10 of Brown's scale and the antigen was extracted by steaming at 100 °C for 60 min. After extraction and cooling, the supernatant was separated by centrifugation and used as antigen. Sterility of the antigen was checked and it was stored at –20 °C until used.

Phenol extract. Phenol extract was prepared following the methods of BUXTON and ALLAN [15].

To the agar washed culture of *S. enteritidis* 5694 SMR adjusted to opacity No. 10, double the quantity of 90% phenol was added and the mixture was heated in a water bath at 68 °C for 30 min with the automatic shaker fixed at 100 cycles per min. The mixture was allowed to cool and centrifuged at 4000 rpm for 15 min in a Remi T23 centrifuge. The clear

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supernatant was separated, mixed to five volumes of pre-cooled ethanol and kept at 4 °C for three hours. The precipitate which had formed was separated by centrifugation at 4000 rpm for 15 min and dissolved to the original volume and dialysed against NSS for 48 hr.

The antigen was tested for sterility and stored at -20 °C until used.

Trichloroacetic acid extract (TCA extract). The procedure described by STAUB [16] was followed. A washed agar culture of *S. enteritidis* 5694 SMR was obtained as before and sedimented in a preweighed centrifuge tube and distilled water was added to five times the weight of the sediment and the mixture was kept at 0 °C for one hour. To this an equal volume of 0.5 N trichloroacetic acid was added and kept at 4 °C for 3 hr.

The mixture was then heated to room temperature and centrifuged in a Remi T23 centrifuge at 4000 rpm for 15 min. The supernatant was removed, the pH adjusted to 6.5 with 0.1 N NaOH and the complex was precipitated with two volumes of ethanol at 4 °C overnight. The sediment was collected by centrifugation and dissolved in distilled water to one-tenth of the original volume. The antigen thus prepared was dialysed against tap water for two days and against distilled water for another two days. The antigen was centrifuged at 6000 rpm for 30 min to remove any possible cell debris and the clear supernatant was collected and stored at -20 °C.

Hypersensitivity test. Twenty-one days after vaccination with live vaccines and live vaccines with complete adjuvant incorporating *S. enteritidis* Se795, *S. typhi-murium* M206, *S. gallinarum* 9R or *S. pullorum* Sp223, seven mice from each group along with unvaccinated control mice were inoculated with saline extract, phenol extract or TCA extract. The eliciting antigen was inoculated in 0.05 ml amounts intradermally in the left hind foot pad after measuring the foot pad with vernier callipers. Into the contralateral (right hind) foot pad 0.05 ml of sterile NSS was injected. The thickness of the foot pads were measured at 5, 24, 48 and 72 hr post-elicitation. The difference in thickness between the antigen foot pad and the saline foot pad constituted the absolute increase in thickness. The arithmetic mean for 7 mice at 5 hr, 6 mice at 24 hr, 5 mice at 48 hr, and 4 mice at 72 hr, post-elicitation was recorded for assessing the hypersensitivity reaction.

At each time point from each group one mouse was sacrificed and the foot pads were preserved in 10% formalin for histological examination. The foot pad was trimmed, embedded in paraffin and sections 5 to 6 µm thick were cut and stained with haematoxylin and eosin.

Clearance of the challenge organism. Groups of mice were vaccinated with the live vaccines and with live vaccines with complete adjuvant incorporating the vaccine strains stated above. On the 21st day post-vaccination all the mice together with a group of unvaccinated controls were challenged with *S. enteritidis* 5694 SMR.

Two mice from each group were sacrificed at 24 and 72 hours and at 6, 9, 12, 15 and 21 days post-challenge. The liver was collected in a preweighed sterile Petri dish under sterile precautions and the weight of the liver was determined. Tenfold dilutions of the liver were made after maceration in a mortar with sterile pestle. From each dilution (at least three dilutions per liver) 0.1 ml aliquots were transferred to a dried MacConkey agar plate (two plates per dilution) with 30 µg of streptomycin. The dropped material was spread with a sterile platinum loop and the plates were incubated at 37 °C overnight. The number of colony forming units (c.f.u.) in each dilution and the number of c.f.u. per g of liver was calculated, taking the arithmetic mean of at least four readings (two readings for each mouse and two mice for each vaccine group). At random the colonies were checked for serological identity.

Results

Results of the hypersensitivity test with saline, phenol and TCA extracts of *S. enteritidis* 5694 SMR as eliciting antigens are shown in Tables I, II and III, respectively. Saline and phenol extracts proved to be poor eliciting antigens when compared to the TCA extract. There was no toxic reaction with TCA extract in unvaccinated control mice. In vaccinated mice, this antigen elicited definite reactions not only at 5 hr, but also at 24, 48 and 72 hr post-elicitation. The difference between the thickness of the antigen foot pad and the saline foot pad was significant. An absolute increase of 0.3 mm (roughly 30% of the original thickness) and above, was considered significant.

Table I

Hypersensitivity reactions to saline extract of S. enteritidis 5694 SMR in mice immunized with different types of vaccines

Vaccine	Absolute increase in thickness of antigen foot pad saline over foot pad in mm at			
	5 hr	24 hr	48 hr	72 hr
	post-elicitation			
<i>S. typhi-murium</i> M206 live vaccine (M206 L)	0.028	0.000	0.020	0.000
with complete adjuvant (M206 LA)	0.000	0.083	0.120	0.000
<i>S. enteritidis</i> Se795 live vaccine (Se795 L)	0.086	0.217	0.140	0.000
with complete adjuvant (Se795 LA)	0.014	0.214	0.220	0.075
<i>S. gallinarum</i> 9R live vaccine (9R L)	0.014	0.050	0.040	0.000
with complete adjuvant (9R LA)	0.014	0.150	0.080	0.025
<i>S. pullorum</i> Sp223 live vaccine (Sp223 L)	0.000	0.066	0.080	0.000
with complete adjuvant (Sp223 LA)	0.000	0.100	0.080	0.000
None	0.000	0.000	0.000	0.000

The figures are the means of post-elicitation readings from seven mice at 5 hr, six mice at 24 hours, five mice at 48 hours and four mice at 72 hours

Table II

Hypersensitivity reactions to phenol extract of S. enteritidis 5694 SMR in mice immunized with different types of vaccine

Vaccines	Absolute increase in thickness of antigen foot pad over saline foot pad in mm at			
	5 hr	24 hr	48 hr	72 hr
	post-elicitation			
M206 L	0.043	0.000	0.120	0.050
M206 LA	0.057	0.083	0.075	0.000
Se795 L	0.043	0.166	0.180	0.050
Se795 LA	0.000	0.214	0.200	0.025
9R L	0.057	0.114	0.060	0.000
9R LA	0.043	0.140	0.075	0.033
Sp223 L	0.014	0.180	0.125	0.000
Sp223 LA	0.014	0.150	0.000	0.000
None	0.000	0.000	0.000	0.000

The figures are the means of post-elicitation readings from seven mice at 5 hr, six mice at 24 hours, five mice at 48 hours and four mice at 72 hours

Table III

Hypersensitivity reactions to trichloroacetic acid extract of S. enteritidis 5694 SMR in mice immunized with different types of vaccine

Vaccine	Absolute increase in thickness of antigen foot pad over saline foot pad in mm at			
	5 hr	24 hr	48 hr	72 hr
	post-elicitation			
M206 L	0.086	0.266	0.220	0.125
M206 LA	0.066	0.240	0.300	0.166
Se795 L	0.150	0.460	0.475	0.333
Se795 LA	0.057	0.450	0.425	0.400
9R L	0.143	0.300	0.260	0.175
9R LA	0.071	0.383	0.280	0.175
Sp223 L	0.066	0.180	0.225	0.100
Sp223 LA	0.157	0.366	0.360	0.250
None	0.000	0.000	0.000	0.000

The figures are the means of post-elicitation readings from seven mice at 4 hr, six mice at 24 hours, five mice at 48 hours, and four mice at 72 hours

At 5 hr post-elicitation, there was a slight reaction in almost all the vaccinated groups but the absolute increase in thickness was less than 0.3 mm.

At 24 hr the mice vaccinated with homologous vaccine groups viz. *S. enteritidis* Se795 live vaccine with complete adjuvant, gave the best reaction closely followed by mice vaccinated with *S. pullorum* Sp223 live vaccine with complete adjuvant and *S. gallinarum* 9R live vaccine with complete adjuvant. The mice receiving *S. typhi-murium* M206 vaccine responded with a poor reaction but the poorest reaction was seen in mice vaccinated with *S. pullorum* Sp223 live vaccine.

At 48 hr post-elicitation the reaction in mice vaccinated with *S. enteritidis* Se795 live vaccine and with live vaccine with complete adjuvant continued to be significant followed by *S. pullorum* Sp223 live vaccine with complete adjuvant. Mice vaccinated with *S. typhi-murium* M206 live vaccine with complete adjuvant induced a better reaction. The reaction in the other groups started to wane except in mice receiving *S. pullorum* Sp223 live vaccine where even with the increase, the reaction remained insignificant.

Histological examination of the foot pad indicated a slight oedema with infiltration of few polymorphonuclear leukocytes at 5 hr post-elicitation. By 24 hr the oedema became extensive with infiltration of polymorphonuclears and monocytes. By 48 hr the former cells were replaced by more and more

monocytes with the oedema persisting. The reaction tended to subside by 72 hr.

Clearance of the challenge organism. Table IV shows the fate of the challenge organism *S. enteritidis* 5694 SMR in the liver of mice inoculated with different vaccines and also in unvaccinated controls, at various intervals after challenge.

Table IV

Clearance of S. enteritidis 5694 SMR from the liver of mice

Vaccine	On days post-challenge						
	1	3	6	9	12	15	21
M206 L	1.8451	2.3222	3.0511	3.0107	2.7818	2.7202	0
M206 LA	1.9294	2.0792	2.4914	2.2553	1.8751	0	0
Se795 L	1.8751	2.1903	2.2900	2.2041	2.0792	2.7404	0
Se795 LA	1.7404	1.9294	2.0792	1.8751	0	0	0
9R L	2.0414	2.4314	3.0986	3.0738	2.9243	2.7853	0
9R LA	2.0212	2.3802	2.4548	2.4150	2.2553	1.6532	0
Sp223 L	2.0969	2.4914	3.1206	3.0828	2.9638	2.8482	0
Sp223 LA	1.9777	2.4065	2.5623	2.4983	2.3222	1.8751	0
None	2.4771	3.6284	4.7202	5.6990	6.4354	NM	NM

The figures represent the number of colony forming units (c.f.u.) per gram of liver, in logarithmic scale

NM = No mice survived, 0 = <20 c.f.u. per g liver

The figures are the means of four readings, two from each of two mice sacrificed at each point of time

In the unvaccinated mice, the challenge organism is able to establish itself quickly and to multiply rapidly in the liver. No mice of the control group survived after 14 days post-challenge and so in this group no further count could be made. The increase was logarithmic as seen at various intervals.

In contrast, vaccinated mice are able to resist the invasion and restrict the multiplication of the challenge organism. The degree of clearance varied from vaccine to vaccine. The mice vaccinated with live vaccine with complete adjuvant were able to clear the challenge organism better than the mice receiving the corresponding live vaccine alone.

Mice vaccinated with the homologous *S. enteritidis* Se795 live vaccine with complete adjuvant were able to clear from the liver the challenge organism *S. enteritidis* 5694 SMR by the 12th day post-challenge, whereas mice vaccinated with heterologous *S. typhi-murium* M206 live vaccine with complete adjuvant could only clear until the 15th day.

At 15 days post-challenge, the degree of clearance by mice vaccinated with homologous live vaccine compares favourably with the degree of clearance

in mice vaccinated with *S. pullorum* Sp223 live vaccine with complete adjuvant and *S. gallinarum* 9R live vaccine with complete adjuvant.

On the 21 day post-challenge, all the surviving mice in all the groups of vaccines were able to clear the challenge organism.

Discussion

Delayed hypersensitivity (DH) has now clearly been understood to be an immunological reaction of acquired cellular resistance [3]. After sensitization by the vaccine, the host reacts with the challenge organism or its products to bring about this reaction inducing an influx of mononuclear cells to the site, perhaps to initiate phagocytosis.

Trichloroacetic acid extract of *S. enteritidis* 5694 SMR proved to be a useful eliciting antigen. There were no toxic reactions and the hypersensitivity reaction was significant and unambiguous. COLLINS and MACKANESS [3] who used a purified culture filtrate as the eliciting antigen were of the opinion that the eliciting antigen should be free from carbohydrates so as to clearly differentiate between an Arthus reaction. DAVIES and KOTLARSKI [17] failed to produce such an effective antigen; instead, they prepared the eliciting antigen by sonicating the organism but since they did not eliminate the carbohydrate, they could not differentiate an Arthus reaction from the delayed type reaction.

The criterion of a good eliciting antigen is that it is not toxic, does not elicit nonspecific reactions in unsensitized control animals and in sensitized animals the reaction must be significant as regards the increase in thickness and in histological pattern. The TCA extract meets well these requirements.

The DH response elicited by TCA extract of *S. enteritidis* 5694 SMR in mice vaccinated with different vaccines correlates with the protection level induced by these vaccines against challenge with *S. enteritidis* 5694 SMR [1] as well as with the clearance rate (Table IV). The DH response in mice vaccinated with *S. typhi-murium* M206 live vaccine with complete adjuvant is less when compared to the protection level induced by this vaccine. This situation needs further attention.

The TCA extract of *S. enteritidis* 5694 SMR could elicit the hypersensitivity response in mice vaccinated with different vaccines. This is further evidence that the expression of cell-mediated immunity is carrier-specific and also explains the cross-protection afforded by these vaccines against challenge with *S. enteritidis* 5694 SMR.

Clearance of the challenge organism is perhaps the test which should be followed to evaluate the potency of any immunizing agent, especially in infections with facultative intracellular parasites. The carrier state of the disease

is much more dangerous to society (population) than the clinical disease as the carrier might be a source of epidemics.

This method of evaluation was initiated by MACKANESS *et al.* [14]. BLANDEN *et al.* [18] proved that the increased clearance was due to cellular immunity and not to antibodies as suggested by JENKIN *et al.* [19]. COLLINS *et al.* [20] clearly demonstrated the differences in the potency of vaccines which otherwise would have been considered equal.

The present results concerning the clearance of the challenge organism indicate a close correlation with the percentage of protection [1], in agreement with the results of COLLINS [10, 13]. The differences in clearance between vaccines and between the hours after challenge are significant when assessed by the variance test. The difference between animals within vaccines remained insignificant thereby pointing to a minimum error.

The close correlation between the protection induced by different vaccines and DH reactions in mice sensitized by these vaccines as well as the clearance of the intracellular facultative parasite from the liver of mice immunized by these vaccines (Table V) suggests that a cell-mediated immune response is involved to a great extent in protection, especially cross-protection. The humoral antibodies, if present, may act complementarily.

Table V

Comparison of protectivity, late allergic reaction and clearance of challenge organisms

Vaccine	Percentage of protection	Delayed hypersensitivity at 48 hr*	Clearance of challenge organism by 12th day**
M206 L	30.77	0.220	2.7818
M206 LA	75.00	0.300	1.8751
Se795 L	91.66	0.475	2.0792
Se795 LA	100.00	0.425	0.0000
9R L	57.14	0.260	2.9243
9R LA	60.00	0.280	2.2553
Sp223 L	38.46	0.225	2.9638
Sp223 LA	87.50	0.360	2.3222
None	0.00	0.000	6.4354

* Absolute increase in thickness of antigen foot pad over saline foot pad in mm

** The figures indicate the number of c.f.u. per g liver; logarithmic scale

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XENOBIOTICS AND SOIL MICROBIOTA AFFECTED BY XENOBIOTIC INTERACTIONS. CHLOROBROMURON AND ITS BREAKDOWN PRODUCT INHIBITING THE GROWTH OF DIFFERENT MICROORGANISMS

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The inhibitory effect of analytical, technical and formulated chlorobromuron (CBU) and its combined inhibitory effect with chlorobromaniline (CBA) were studied on 12 soil microorganisms at 10 and 100 ppm concentrations. *Geotrichum candidum*, *Aureobasidium pullulans*, *Candida utilis*, *Cryptococcus albidus* var. *diffluens*, *Kluyveromyces marxianus*, *Rhodotorula rubra* and *Rhizobium meliloti* were severely inhibited at 10 ppm CBU concentration. CBU at each concentration inhibited the tested microorganisms except for *Pseudomonas aeruginosa*, the growth of which was slightly stimulated. The four impurities detected in the technical and formulated products by GC and TLC methods increased the inhibitory effect on *Prototheca wickerhamii*, *Geotrichum candidum*, *Torulopsis candida*, *Bacillus subtilis* var. *niger* and *Rhizobium japonicum*. *Prototheca wickerhamii* and *Kluyveromyces marxianus* were equally sensitive to CBU and CBA. *Geotrichum candidum*, *Bacillus subtilis* var. *niger* and *Cryptococcus albidus* var. *diffluens* were more inhibited by CBA. The growth of *Rhizobium meliloti* was stimulated by CBA.

Chlorobromuron (Fig. 1) is an important and widely used representative of urea herbicides. Earlier we have studied its fate and decomposition in soils under experimental conditions [1–5], and its effect on the soil microorganisms.

In this investigation the aim was to compare the inhibitory effect of analytically pure, technical and formulated chlorobromuron and its breakdown product, chlorobromaniline on 12 different microbial strains, to obtain data for the “side-effects” of pesticides on “non target” soil microorganisms.

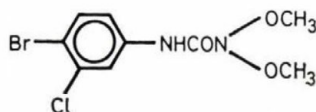


Fig. 1. Chlorobromuron

Materials and methods

Chlorobromuron (CBU) samples different in purity were analysed by thin layer and gas-liquid chromatographic methods applied for detecting CBU and its breakdown products [2].

From every CBU sample (5 ml CBU/ml in acetone) 500 μ g was applied to 0.25 mm thick 20 $\times$ 20 cm Kieselgel HF 254 plates and developed by a mixture of benzene : acetone 9 : 1. The spots were detected in UV light.

The conditions for the gas chromatographic detection of CBU and its impurities were, detector type: flame ionization; column: 180 cm long, glass, with 3 mm internal diameter, packed with 3% SE 30, on Chromosorb W 60–80 mesh; injector temperature: 200 °C; column temperature: 180 °C; detector temperature: 200 °C carrier gas: N₂ 40 ml/min; sensitivity: 3×10^{-9} .

To study the effect of analytical, technical and formulated CBU on microorganisms, 12 strains (shaken liquid culture) were incubated at 26–28 °C in a nutrient broth (Oxoid CM 1), containing 10 or 100 ppm of different CBU samples. Untreated samples were the controls. The starting microbe number was set to 10^6 microbes/ml. Growth was followed turbidimetrically at 600 nm wavelength. The experiments were carried out with 5 parallels in 2 repetitions. Mean standard deviations and mean variation coefficients were calculated for each strain. To decide whether the turbidimetric method gives correct data for the microbe number of every species, the standard deviations were subjected to the Bartlett test.

The test organisms were, *Aureobasidium pullulans* 76/128, *Geotrichum candidum* 76/K 35 filamentous microscopic fungi; *Prototheca wickerhamii* 9 yeast-like alga; *Candida utilis* 472, *Cryptococcus albidus* var. *diffluens* P7, *Kluyveromyces marxianus*, *Rhodotorula rubra* 1165, *Torulopsis candida* S 579 yeasts; *Pseudomonas aeruginosa*, *Bacillus subtilis* var. *niger* KJ 51, *Rhizobium japonicum* 001/NPN and *Rhizobium meliloti* TA 402. The combined effect of CBU and its hydrolysis product, chlorobromaniline (CBA) on microorganisms were investigated as described above. The following total CBU + CBA concentrations were used for the selected six organisms: *P. wickerhamii*, *G. candidum*, *C. albidus* var. *diffluens*, *K. marxianus*, *B. subtilis* var. *niger* and *R. meliloti*: 50, 10, 100, 50, 100 and 100 ppm, respectively. The applied CBU–CBA ratios were 0:10; 2:8; 4:6; 6:4; 8:2; 10:0. The cultures were incubated and shaken at 26–28 °C for 16 hr and the cell number was determined turbidimetrically as above. The mean standard deviation and mean variation coefficient were calculated for the data.

Results and discussion

The analytical CBU sample was pure according to the TLC method, but the technical product and Maloran contained four impurities. The R_F values are shown in Table I. Using GLC, the pure CBU gave one peak, the technical product gave four peaks with retention times 2.2; 3.7; 5.3 and 5.9 min. In Maloran only one impurity (3.7 min) was detected in an amount comparable to technical CBU, the others were found in smaller amounts.

Table I
 R_F values of CBU and its impurities

	Analytical CBU	Technical CBU	Maloran
1		0.0	0.0
2		0.21	0.21
3		0.32	0.32
4	0.56	0.56	0.56
5		0.68	0.68

The GLC curves can be seen in Figs 2, 3 and 4. Considering the data with respect to TLC and GC, the technical CBU and Maloran contained four impurities each, in different quantities.

The effect of CBU on twelve strains is presented in Table II.



Fig. 2. GLC curve of analytical CBU



Fig. 3. GLC curve of technical CBU

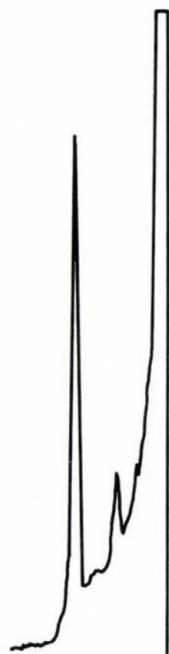


Fig. 4. GLC curve of Maloran 50 WP

From the data of Table II the following conclusion can be drawn: *A. pullulans* is very sensitive to CBU even at 10 ppm concentration; the inhibition is more marked at 100 ppm. The impurities, present in the other samples do not influence the inhibition, there is no significant difference between the effect of the analytical and technical samples.

In the case of *P. wickerhamii* 10 ppm analytical CBU has no inhibitory effect, while the same amount of technical CBU and Maloran significantly inhibit the growth. At 100 ppm concentration there is no difference in the inhibition, suggesting that this strain is sensitive to impurities in small quantities.

G. candidum behaved similarly to *A. pullulans*. The inhibitory effect of CBU is considerable at 10 ppm concentration and even more at 100 ppm. There is no significant difference between the inhibitory effect of analytical and technical CBU, so this strain is rather sensitive to CBU and hardly to the impurities.

C. utilis and *C. albidus* var. *diffluens* are inhibited by 10 ppm CBU and the inhibition is more marked at 100 ppm. Technical CBU and Maloran show the same but somewhat higher inhibition.

Treating strain *K. marxianus* with either 10 or 100 ppm analytical CBU, 10 ppm technical CBU and Maloran, inhibition was the same. At 100 ppm

Table II
Effect of chlorobromuronon soil microorganisms

Strain	Treatment	Growth in per cent of the control	Significant deviation from			Incuba- tion time (hr)	Varia- tion coeffi- cient
			control	anal	techn		
<i>A. pullulans</i>	anal 10 ppm	44.8	× × ×			38	9.4
	anal 100 ppm	12.8	× × ×				
	techn 10 ppm	38.3	× × ×	—			
	techn 100 ppm	13.6	× × ×	—			
	Maloran 10 ppm	113.8	+ ×	+ × × ×	+ × × ×		
	Maloran 100 ppm	34.7	× × ×	× ×	+ × ×		
<i>G. candidum</i>	anal 10 ppm	93.3	—			38	6.6
	anal 100 ppm	23.3	× × ×				
	techn 10 ppm	72.8	× × ×	× × ×			
	techn 100 ppm	20.3	× × ×	—			
	Maloran 10 ppm	75.4	× × ×	× ×	—		
	Maloran 100 ppm	32.6	× × ×	—	+ —		
<i>P. wicker- hamii</i>	anal 10 ppm	57.4	× × ×			16	12.8
	anal 100 ppm	18.6	× × ×				
	techn 10 ppm	45.3	× × ×	—			
	techn 100 ppm	16.7	× × ×	—			
	Maloran 10 ppm	37.7	× × ×	×	—		
	Maloran 100 ppm	37.8	× × ×	×	—		
<i>C. utilis</i>	anal 10 ppm	64.7	× × ×			24	4.6
	anal 100 ppm	25.9	× × ×				
	techn 10 ppm	66.1	× × ×	—			
	techn 100 ppm	17.8	× × ×	×			
	Maloran 10 ppm	58.2	× × ×	×	×		
	Maloran 100 ppm	17.5	× × ×	×	—		
<i>C. albidus</i> var. <i>diffluens</i>	anal 10 ppm	78.9	× × ×			24	4.6
	anal 100 ppm	45.5	× × ×				
	techn 10 ppm	83.9	× ×	—			
	techn 100 ppm	30.3	× × ×	× ×			
	Maloran 10 ppm	63.6	× × ×	× ×	× × ×		
	Maloran 100 ppm	40.8	× × ×	—	×		
<i>K. marxianus</i>	anal 10 ppm	70.8	× × ×			20	5.1
	anal 100 ppm	74.8	× × ×				
	techn 10 ppm	65.4	× × ×	—			
	techn 100 ppm	5.5	× × ×	× × ×			

× significant at 95.0%
 × × significant at 99.0%
 × × × significant at 99.9%

Cont. table II.

Strain	Treatment	Growth in per cent of the control	Significant deviation from			Incuba- tion time (hr)	Vari- ation coeffi- cient
			control	anal	techn		
<i>R. rubra</i>	Maloran 10 ppm	86.7	×	×	× × ×	20	6.8
	Maloran 100 ppm	10.7	× × ×	× × ×	—		
	anal 10 ppm	68.1	× × ×				
	anal 100 ppm	38.5	× × ×				
	techn 10 ppm	70.1	× × ×	—			
	techn 100 ppm	21.3	× × ×	× ×			
<i>T. candida</i>	Maloran 10 ppm	74.9	× × ×	—	—	24	10.2
	Maloran 100 ppm	32.3	× × ×	—	×		
	anal 10 ppm	90.3	—				
	anal 100 ppm	50.1	× × ×				
	techn 10 ppm	70.9	× × ×	×			
	techn 100 ppm	42.8	× × ×	—			
<i>P. aeruginosa</i>	Maloran 10 ppm	68.1	× × ×	× ×	—	16	10.7
	Maloran 100 ppm	43.9	× × ×	—	—		
	anal 10 ppm	78.8	×				
	anal 100 ppm	76.7	× ×				
	techn 10 ppm	70.1	× ×	—			
	techn 100 ppm	92.7	—	×			
<i>B. subtilis</i> var. <i>niger</i>	Maloran 10 ppm	87.5	—	—	× ×	16	10.6
	Maloran 100 ppm	84.3	×	—	—		
	anal 10 ppm	114.9	—				
	anal 100 ppm	82.1	×				
	techn 10 ppm	106.2	—	—			
	techn 100 ppm	17.1	× × ×	× × ×			
<i>R. japonicum</i>	Maloran 10 ppm	109.2	—	—	—	16	0.95
	Maloran 10 ppm	18.4	× × ×	× × ×	—		
	anal 10 ppm	105.5	× × ×				
	anal 100 ppm	105.4	× × ×				
	techn 10 ppm	105.8	× × ×	—			
	techn 100 ppm	93.4	× × ×	× × ×			
<i>R. meliloti</i>	Maloran 10 ppm	109.1	× × ×	× × ×	× × ×	38	6.8
	Maloran 100 ppm	97.3	× × ×	× × ×	× × ×		
	anal 10 ppm	66.3	× × ×				
	anal 100 ppm	38.1	× × ×				
	techn 10 ppm	58.5	× × ×	—			
	techn 100 ppm	40.1	× × ×	—			
	Maloran 10 ppm	75.8	× × ×	—	× ×		
	Maloran 100 ppm	58.5	× × ×	× × ×	× ×		

concentration of the latter samples the inhibition was intensive, which can be explained by the impurities present.

R. rubra is very sensitive to CBU; all the three CBU samples inhibited it considerably at 10 ppm. This effect increased at 100 ppm, but in this concentration the analytical sample is less inhibitory than are the other two.

T. candida was not significantly inhibited by 10 ppm analytical CBU, only by 100 ppm. The effect of technical CBU and Maloran was considerable at 10 ppm concentration, and even more so at 100 ppm. There was no significant difference in the effect of the three various CBU preparations at 100 ppm. So this strain is not sensitive to CBU at low concentration, but it is inhibited by the impurities present in the technical CBU.

There were hardly any differences in the effect of CBU in the case of *P. aeruginosa*. This bacterium was inhibited by CBU but the inhibition apparently did not depend on the quality or quantity of CBU in the concentration interval investigated.

B. subtilis var. *niger* did not suffer significant damage from 10 ppm CBU of whatever kind. At higher concentration (100 ppm) analytical CBU already inhibited growth but two others had a stronger effect.

The most interesting was the effect of CBU on *R. japonicum*, where the activity of CBU was low in each treatment, with the deviation from the control being less than 10%, but the deviation was positive with analytical CBU at 10 and 100 ppm and with technical CBU and Maloran at 10 ppm. The deviation caused by the technical CBU and Maloran at 100 ppm is negative. We assume that CBU stimulates *R. japonicum* but this is compensated by the inhibitory effect of the impurities.

The soil bacterium *R. meliloti* is inhibited by all the three CBU samples at 10 ppm and more intensely at 100 ppm. At 10 and 100 ppm we could not find a difference between analytical and technical CBU. Maloran in both concentrations had a weaker inhibitory effect than the technical and analytical CBU samples.

Summing up, CBU inhibits the tested soil microorganisms practically at each concentration except for strain *R. japonicum*, where CBU slightly stimulated growth. Most of the investigated soil microorganisms (*A. pullulans*, *G. candidum*, *C. utilis*, *C. albidus* var. *diffluens*, *K. marxianus*, *R. rubra* and *R. meliloti*) were severely inhibited by 10 ppm of CBU, the usual concentration applied in agricultural practice. For strains *P. wickerhamii*, *G. candidum*, *T. candida*, *B. subtilis* var. *niger* and *R. japonicum* the inhibitory effect of technical CBU and Maloran was stronger than that of analytical CBU. This agrees well with the observation that technical CBU and Maloran contain impurities responsible for the stronger inhibitory effect. The fact that the inhibitory effect of Maloran was mostly slighter than that of technical CBU, was surprising at first, but Maloran contains less impurities than the

technical CBU. That is why Maloran was less inhibitory than technical CBU in most experiments.

The standard deviations were analyzed by the Bartlett test (Table III); this showed that the standard deviation of the results of the turbidimetric method was not the same for all the strains at a significance level of 99.0%. The deviation for the *C. utilis* and *R. japonicum* strains was significantly lower, and for *P. aeruginosa*, significantly higher, than the average. From Table IV it can be seen that *P. wickerhamii* is equally sensitive to CBA and CBU,

Table III

Analysis of the standard deviation by the Bartlett test

Strain	Incubation (hr)	$\chi^2 = \frac{SQ}{S_e}$	$\chi^2 1\%$
<i>A. pullulans</i>	38	1.83	0.554
<i>G. candidum</i>	38	15.58	
<i>P. wickerhamii</i>	16	5.01	
<i>C. utilis</i>	24	0.44	
<i>C. albidus</i> var. <i>diffluens</i>	24	0.95	
<i>K. marxianus</i>	20	7.51	18.55
<i>T. rubra</i>	20	4.63	
<i>T. candida</i>	24	7.55	
<i>P. aeruginosa</i>	16	25.75	
<i>B. subtilis</i> var. <i>niger</i>	16	12.16	
<i>R. japonicum</i>	16	0.82	0.872
<i>R. meliloti</i>	20	7.51	

$$\chi^2 = 263.55, \chi^2_{FG 16 p 0.1} = 39.3$$

Table IV

Combined effect of CBU and CBA

CBU%	CBA%	Growth in per cent of the control					
		<i>G. candidum</i>	<i>P. wickerhamii</i>	<i>C. albidus</i> var. <i>diffluens</i>	<i>K. marxianus</i>	<i>B. subtilis</i> var. <i>niger</i>	<i>R. meliloti</i>
100	0	50.62	86.03	45.35	73.31	113.97	81.41
80	20	59.49	83.81	29.82	75.54	113.29	86.03
60	40	50.50	80.32	28.71	79.00	93.71	96.84
40	60	50.06	81.27	22.82	61.57	18.53	101.46
20	80	46.39	86.03	18.24	65.45	18.91	124.42
0	100	48.24	70.12	11.17	64.77	3.50	137.79
s		0.024	0.004	0.031	0.003	0.019	0.007
v		9.58	3.06	11.65	2.43	17.90	1.73

while the growth of *G. candidum* is inhibited more by CBA. As regards the effect of CBU and CBA, considerable differences were noticed in the case of *C. albidus* var. *diffluens*, where CBA had a remarkable inhibitory effect. The effect of CBU and CBA was similar on *K. marxianus*. *B. subtilis* var. *niger* was very sensitive to CBA at 10 ppm and unaffected by CBU even at 100 ppm. The inhibition of *R. meliloti* by 10 ppm CBU can be compensated by the presence of 30–40% CBA and the growth can even be stimulated by increasing the concentration of CBA.

Figure 5 shows three examples of the effect of CBU and CBA. For *P. wickerhamii* CBU and CBA have the same effect, *C. albidus* var. *diffluens* is much more sensitive to CBA than to CBU, and *R. meliloti* is inhibited by CBU and stimulated by CBA.

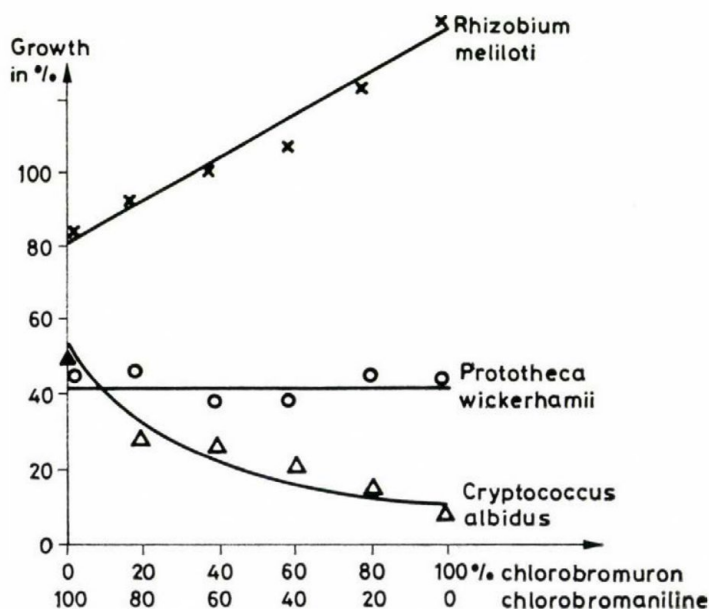


Fig. 5. Combined effect of CBU and CBA

Our earlier investigations [1, 6–9] proved the importance of studying the xenobiotic interactions on soil microflora, and from our present results we again conclude that in determining the microbicidal effect of a pesticide we must take into consideration the impurities and breakdown products, as well as their combined effects, which may influence the effect of a pesticide on soil microorganisms.

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ISOLATION AND CHARACTERIZATION OF STREPTOMYCES SCABIES STRAINS FROM SCAB LESIONS OF POTATO TUBERS. DESIGNATION OF THE NEOTYPE STRAIN OF STREPTOMYCES SCABIES

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A total of 452 strains of *Streptomyces scabies* were separated from among the 465 streptomycete strains which were isolated from scab lesions on potato tubers. On the basis of comparative cultural-morphological and physiological investigation on 86 selected representative strains, KE-5/S: 4-97 and KE-5/S: 1-2 are proposed as neotype and type strains of *S. scabies* and *S. scabies* var. *achromogenes* var. nov., respectively.

Potato scab disease being responsible for a considerable economic loss in agriculture, many studies have dealt with the disease and with the actinomycetes and other microbes producing the infection. Among the streptomycetes isolated from infected potato tubers, *Streptomyces scabies* seems to be the most common potato scab producing organism. Although *S. scabies* is one of the most extensively studied species of *Streptomyces*, many uncertainties exist as to its taxonomic position. The original strain is no longer extant and we have certain "authentic" strains of very different diagnostic features. According to HÜTTER [1] strains of *S. scabies* (*Oospora scabies* Thaxter 1891; *Actinomyces scabies* Güssow 1914; *S. scabies* Waksman et Henrici 1948) produce, presumably, gray coloured mature aerial mycelia, spiral sporophores, smooth spores and melanoid pigments. *S. scabies* IPV 96, which shows this combination of diagnostic features, was clustered with similar strains of *S. tendae*, *S. aurantiogriseus*, *S. galbus*, etc., together into one single species designated as *S. tendae* by HÜTTER [1]. *S. scabies* CBS strain de Vries and strain ATCC 10246 (Cornell Univ. Nr 54) belong to the *Griseus*-group, and they are members of other species. In the 8th edition of Bergey's Manual [2] *S. scabies* was listed within the group of "species incertae sedis" among those of which the type strains are not extant. The "type" strain IMRU 3018, that was proposed by WAKSMAN [3] has not been accepted because of its micromorphology which is not characteristic of the species.

The purpose of this paper is to report the results of a comparative study on the species-composition of streptomycete populations of typical lesions of potato scab and to select and designate a neotype strain from a large and relatively homogeneous collection of freshly isolated strains of *S. scabies*.

Materials and methods

Isolation procedures. Strains of streptomycetes were isolated from potato scab lesions (May, 1977) on casein-starch agar [4] and on glycerol-arginine agar [5]. Scabbed tubers of different potato sorts were selected from the experimental material of the Potato Research Department of the Agricultural University, Keszthely, Hungary. Individual scab lesions were removed separately and aseptically from the surface of tubers, then mechanically homogenized, diluted and plated. The colonies developed were picked at random and transferred to slants of oatmeal agar. The isolated cultures after incubation at 28 °C for 1-2 weeks were maintained on oatmeal agar slants in a refrigerator at 3 °C.

Description of strains. Methods (morphological sections of SHIRLING and GOTTLIEB; colour wheals developed by TRESNER and BACKUS; determination of carbon utilization pattern on PRIDHAM and GOTTLIEB's basal salts medium; indicator pigments detected by observing the effect of 0.05 N NaOH and 0.05 N HCl on the colour of the reverse side of colony, etc.) and media (yeast extract-malt extract agar; oatmeal agar; inorganic salts-starch agar; glycerol-asparagine agar; tyrosine agar; peptone-yeast-iron agar) employed by the collaborators of the International Streptomyces Project (ISP) were used [6]. Nitrite produced from nitrates in nitrate broth was demonstrated by the Griess-Ilosvay method. Hydrogen sulfide test was studied by the method recommended by KÜSTER and WILLIAMS [7]. The hydrolysis of Tweens was tested by the methods described by SIERRA [8]. Hydrolysis of starch and cellulose, decomposition of gelatin, urease production, utilization of nitrate as sole source of nitrogen, growth on paraffin and in the presence of 3-10% NaCl were tested by methods used in our laboratory [9].

Results

A total of 465 streptomycete strains was isolated from seven scab lesions of seven tubers representing two different sorts of potato (Tables I and II). The species composition of streptomycete populations of the seven scab lesions were analysed by investigating 41, 31, 104, 104, 46, 69 and 70 strains, respectively. The populations of two scab lesions (KE-5/S: 1 and KE-5/S: 4) represented by 41 and 104 isolated strains, respectively, were homogeneous, being composed only of the members of *S. scabies*. When, however, we compared these two scabies populations on the basis of ISP-criteria, it was obvious that they differ in their melanoid-pigment production. All the strains representing the colonizers or more exactly the invaders from the first scab lesion (KE-5/S: 1) were unable to produce melanoids either on peptone-iron agar or on tyrosine agar. On the other hand, all *S. scabies* strains that originated from the scab lesion of tuber KE-5/S: 4 proved to be melanoid-positive on both media. The relation of melanoid-positive (M^+) to negative (M^-) strains varied with the lesions. Few M^- strains were associated with M^+ forms in the case of lesion KE-5/S: 3; 30 strains (group A) were M^+ and only one single (group B) was M^- . Similarly, group A of lesion KE-9/S: 1 comprised 92 M^+ strains while the groups C and D of this lesion only 1 M^- variant each which latter differed in the colour of the produced soluble pigments. In the *S. scabies* population (total 68 strains) of lesion KE-9/S: 3, the number of M^+ variants was 14.

Streptomycetes belonging to different species not identical with *S. scabies* occurred only sporadically in the individual lesions: e.g. from lesion KE-9/S: 3 one (group C) and from the lesion KE-9/S: 4 two such strains (groups

Table I

Total number, selected groups and some diagnostic features of streptomycete strains isolated from scab lesions of potato tubers

Sort of potato Designation of investigated tuber Total number of isolated strains			KE-5/S 1 41	KE-5/S 3 31	KS-5/S 4 104	KE-9/S 1 104				KE-9/S 2 46	KE-9/S 3 69			KE-9/S 4 70		
Selected groups of similar or identical strains			A	A B	A	A	B	C	D	A B	A	B	C	A	B	C
Number of strains belonging to individual groups			41	30 1	104	92	10	1	1	45 1	54	14	1	68	1	1
Melanoid-pigment production on tyrosine agar	+		0	30 0	104	92	0	0	0	45 0	0	14	0	68	1	0
	—		41	0 1	0	0	10	1	1	0 1	54	0	1	0	0	1
Melanoid-pigment production on peptone-iron agar	+		0	30 0	104	92	0	0	0	45 0	0	14	0	68	0	0
	—		41	0 1	0	0	10	1	1	0 1	54	0	1	0	1	1
Colour of substrate mycelium	yellow-brown		41	30 1	104	92	10	1	1	45 1	54	14	1	68	1	1
	other		0	0 0	0	0	0	0	0	0 0	0	0	0	0	0	0
Response of substrate mycelium pigment to pH change	+		0	0 0	0	0	0	0	0	0 0	0	0	0	0	0	0
	—		41	30 1	104	92	10	1	1	45 1	54	14	1	68	1	1
Colour of the aerial mycelium (spores: en masse)	gray		41	30 1	104	92	0	1	1	45 0	54	14	0	68	1	0
	yellow		0	0 0	0	0	10	0	0	0 0	0	0	0	0	0	0
	green		0	0 0	0	0	0	0	0	0 1	0	0	0	0	0	0
	white		0	0 0	0	0	0	0	0	0 0	0	0	1	0	0	0
	red		0	0 0	0	0	0	0	0	0 0	0	0	0	0	0	1
Colour of the soluble pigments	colourless		41	30 1	104	92	0	1	0	45 0	54	14	0	68	1	0
	yellowish		0	0 0	0	0	2	0	1	0 1	0	0	1	0	0	1
	brownish		0	0 0	0	0	8	0	0	0 0	0	0	0	0	0	0
Sporophore morphology	Spira		41	30 1	104	92	10	1	1	45 1	54	14	1	68	1	1
	Rectus-flexibilis		0	0 0	0	0	0	0	0	0 0	0	0	0	0	0	0

Table II

Occurrence and number of isolated and selected representative strains of *Streptomyces* spp.

Scab lesion No.	Selected group	Species	Total no. of isolated strains	No. of selected representative strains
KE-5/S: 1	A	<i>S. scabies</i> var. <i>achromogenes</i>	41	10
KE-5/S: 3	A	<i>S. scabies</i>	30	10
	B	<i>S. scabies</i> var. <i>achromogenes</i>	1	1
KE-5/S: 4	A	<i>S. scabies</i>	104	15
KE-9/S: 1	A	<i>S. scabies</i>	92	10
	B	<i>S. flavidovirens</i>	10	4
	C	<i>S. scabies</i> var. <i>achromogenes</i>	1	0
	D	<i>S. scabies</i> var. <i>achromogenes</i>	1	0
KE-9/S: 2	A	<i>S. scabies</i>	45	10
	B	<i>Streptomyces</i> sp.	1	0
KE-9/S: 3	A	<i>S. scabies</i> var. <i>achromogenes</i>	54	10
	B	<i>S. scabies</i>	14	10
	C	<i>Streptomyces</i> sp.	1	0
KE-9/S: 4	A	<i>S. scabies</i>	68	10
	B	<i>Streptomyces</i> sp. I	1	1
	C	<i>Streptomyces</i> sp. II	1	0

B and C) were isolated (Tables I and II). Among the "alien" species only one proved to be a true colonizer of the potato tuber and a frequent member of a lesion-community (KE-9/S: 1, group B). All this shows that although the M⁻ variants of *S. scabies* usually occur together with their typical M⁺ forms, practically "pure" M⁻ *S. scabies* populations can be isolated from certain scab lesions.

All isolated M⁺ and M⁻ strains of *S. scabies* produce heavy developed, grey mature aerial mycelium (spores en masse), spiral sporophores and yellow-brown substrate mycelium pigments which do not show indicator character. All strains, investigated by electronmicroscopy, produce smooth spores. Eighty-six freshly isolated representative strains of *S. scabies* (M⁺: 66; M⁻: 21) were selected for further investigation. They all utilized glucose, xylose, fruc-

Table III

Utilization of 16 carbon compounds by 86 selected representative *S. scabies* and *S. scabies* var. *achromogenes* strains isolated from scab lesions of different potato tubers

Sort of potato		KE-5/S	KE-5/S		KE-5/S	KE-9/S	KE-9/S		KE-9/S
Designation of investigated tuber		1	3		4	1	2		4
Designation of the group of isolated strains		A	A	B	A	A	A	B	A
Melanoid-pigment production		M ⁻	M ⁺	M ⁻	M ⁺	M ⁺	M ⁻	M ⁺	M ⁺
Total number of selected representative strains		10	10	1	15	10	10	10	10
Glucose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Fructose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Xylose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Arabinose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Rhamnose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Sucrose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Raffinose	positive	10	10	1	14	10	10	10	10
	negative	0	0	0	1	0	0	0	0
Melibiose	positive	10	10	1	15	9	10	10	10
	negative	0	0	0	0	1	0	0	0
Lactose	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Maltose	positive	10	9	1	15	10	10	10	10
	negative	0	1	0	0	0	0	0	0
Inositol	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Mannitol	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Dulcitol	positive	0	0	0	0	0	0	0	0
	negative	10	10	1	15	10	10	10	10
Starch	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Inulin	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
Dextrin	positive	10	10	1	15	10	10	10	10
	negative	0	0	0	0	0	0	0	0
None	positive	0	0	0	0	0	0	0	0
	negative	10	10	1	15	10	10	10	10

tose, arabinose, rhamnose, lactose, inositol, mannitol, sucrose, starch, inulin and dextrin (Table III). Table IV shows some physiological properties of these strains. It is seen that the members of this large *S. scabies* population of heterogeneous origin show a high degree of similarity, and their sensitivity to antibiotics and antibacterial substances was slightly variable (Table V).

Strain KE-5/S: 4-97 was selected from the population of lesion KE-5/S: 4 as the neotype strain of *S. scabies*. A short description of strain KE-5/S: 4-97 on the basis of ISP methods and criteria is as follows.

Spore chain morphology: Section Spirales. Mature spore chains generally long, often with more than 50 spores per chain. This morphology is seen on oatmeal agar, salts-starch agar and glycerol-asparagine agar. Spore surface: smooth.

Table IV

Selected physiological properties of 86 representative strains of S. scabies

	Total number of strains		
	Positive	Questionable	Negative
Hydrolysis of starch	86	0	0
Hydrolysis of Tween 40	86	0	0
Hydrolysis of Tween 60	86	0	0
Hydrolysis of Tween 80	32	54	0
Hydrolysis of cellulose	0	14	72
Urease	86	0	0
Decomposition of gelatin	86	0	0
Nitrate reduction	74	9	3
Growth in 3% NaCl	86	0	0
6% NaCl	86	0	0
7% NaCl	0	86	0
8% NaCl	0	0	86
Hydrogen sulfide from			
peptone	86	0	0
L-cysteine	86	0	0
Utilization of nitrate as sole			
source of nitrogen	86	0	0
Growth on paraffin	0	0	86
Growth at 37 °C	86	0	0
40 °C	25	0	61
42 °C	0	0	86
45 °C	0	0	86

Table V

Sensitivity to antibiotics and antibacterial substances of representative strains of S. scabies and S. scabies var. achromogenes

	Zones of inhibition in mm	No. of strains		Zones of inhibition in mm	No. of strains
Penicillin (3 IU)	No inhibition	46	Polymyxin-B (15 µg)	1 > 1	46 0
Oxacillin (10 µg)	No inhibition	46	Erythromycin (10 µg)	< 8 8—12 > 12	1 41 4
Methicillin (20 µg)	No inhibition	46	Superseptyl (400 µg)	No inhibition	46
Chloramphenicol (30 µg)	< 17 17—21 > 21	2 43 1	Nitrofurantoin (300 µg)	< 12 12—15 > 15	5 36 5
Streptomycin (30 µg)	< 11 11—14 > 14	3 43 0	Chlortetracycline (30 µg)	< 10 10—14 > 14	3 83 0
Oleandomycin (30 µg)	< 6 6—10 > 10	3 41 2	Oxytetracycline (30 µg)	< 12 12—15 > 15	2 83 1
Tetracycline (30 µg)	< 8 8—10 > 10	0 42 4	Vancomycin (50 µg)	< 12 12—15 > 15	3 82 1
Neomycin (100 µg)	< 8 8—10 > 10	0 45 1	Kanamycin (30 µg)	< 10 10—12 > 12	1 84 1
Spiramycin (30 µg)	< 10 10—15 > 15	1 84 1	Gentamicin (20 µg)	< 6 6—8 > 8	0 86 0
Ampicillin (20 µg)	< 8 8—12 > 12	0 86 0	Carbenicillin (50 µg)	< 10 10—12 > 12	5 81 0
Colistin (20 µg)	No inhibition	86	Nystatin (100 IU)	No inhibition	86
Lincomycin (10 µg)	< 5 5—8 > 8	4 75 7	Gramurin	No inhibition	86
Cephalosporin (10 µg)	< 5 5—8 > 8	3 83 0			
Pristinamycin (10 µg)	< 10 10—12 > 12	2 81 3			
Nalidixic acid (30 µg)	No inhibition	86			
Paromomycin (50 µg)	< 6 6—10 > 10	1 8 1			

Colour of colony: Aerial mass colour in the Grey colour-series on oatmeal agar, salts-starch agar and glycerol-asparagine agar.

Reverse side of colony: no distinctive pigment (Yellow-brown colour-series); substrate pigment is no pH indicator.

Colour in medium: Melanoid pigments formed in peptone-yeast-iron agar and tyrosine agar. Pigments other than melanoids not formed in yeast-malt agar, oatmeal agar, salts-starch agar or glycerol-asparagine agar.

Carbon utilization: D-glucose, L-arabinose, D-xylose, D-fructose, rhamnose, sucrose, i-inositol, D-mannitol and raffinose are utilized for growth.

The designated type strain — strain KE-5/S: 1-2 — of *S. scabies* var. *achromogenes* var. nov. differs from the neotype strain of *S. scabies* in that it does not produce melanoid pigments either in peptone-yeast-iron agar or in tyrosine agar.

Strains KE-5/S: 4-97 and KE-5/S: 1-2 were deposited in the American Type Culture Collection.

Ten strains which were isolated from the scab lesion KE-9/S: 1 and clustered together into the group B (Table II) proved to be members of *S. flavidovirens* according to HÜTTER's identification key [1]. Their C-source utilization spectrum, however, differs from that of the type strain of *S. flavidovirens*. Because of their frequent occurrence in the above-mentioned scab lesion, four representative strains (KE-9/S: 1-57; -49; -77 and -68) were selected and subjected to further investigation. They showed practically identical cultural-morphological and physiological diagnostic features: 1. Spore chain morphology: Section Spirales. 2. Spore surface: smooth. 3. Aerial mass colour is in the Yellow colour-series. 4. Reverse side of colony: no distinctive pigments on diagnostic media. 5. Melanoid pigment not formed in peptone-yeast-iron agar and tyrosine agar. 6. Carbon utilization: D-glucose, D-xylose, D-fructose, L-arabinose, i-inositol, D-mannitol, raffinose, maltose, starch and dextrine are utilized for growth. No growth or only traces of it on sucrose, rhamnose, melibiose, inulin and dulcitol. The utilization of lactose is doubtful. Hydrolysis of starch, Tween 40, 60 and 80, urease test, decomposition of gelatin, nitrate reduction, growth in 10% NaCl, hydrogen sulfide production from peptone and L-cysteine, growth on paraffin and at 42 °C are all positive. No growth on cellulose and at 45 °C. The utilization of nitrate as the sole source of nitrogen is doubtful. They are resistant to penicillin, oxacillin, methicillin, oleandomycin, erythromycin, nitrofurantoin, ampicillin, colistin, lincomycin, cephalosporin, nalidixic acid, carbenicillin, nystatin and gramurin in the concentration tested. They were sensitive to chloramphenicol, streptomycin, tetracycline, neomycin, chlortetracycline, oxytetracycline, vancomycin, kanamycin, spiramycin, paromomycin, and gentamicin.

Discussion

S. scabies is a member of the large and widely distributed "Diastatochromogenes group" [10] which comprises such species as *S. bottropensis* Waksman, *S. diastatochromogenes* (Krainsky) Waksman and Henrici, *S. eurythermus* Corbaz et al., *S. griseosporus* Niida and Ogasawa, *S. neyagawaensis* Yamamoto et al., *S. versipellis* Oliver et al., etc. Further research is needed to clarify the taxonomic relationship between *S. scabies* and other members of the Diastatochromogenes group. Because both the typical M⁺ forms and the deficient M⁻ variants of *S. scabies* occur as saprophytes, freely in the soil, it seems that among the members of the widely distributed Parvullus group (*S. chibaensis* Suzuki et al., *S. corchorusii* Ahmad and Bhuiyan, *S. libani* Baldacci and Grein, *S. lydicus* De Boer et al., *S. nigellus* Prokop, *S. olivaceiscleroticus* Pridham, *S. parvullus* Waksman and Gregory, *S. pristinaespiralis* Maney et al., *S. thermoflavus* Pridham, etc.) which can be characterized, except for melanoid-pigment production, with the ISP diagnostic features of *S. scabies*, one or more species may be identical with *S. scabies* var. *achromogenes*. Still, the true systematic position of *S. scabies* within the very large and complex assemblage of the "grey coloured streptomycetes" is still uncertain.

The unaccepted "type strain" of WAKSMAN (IMRU 3018 [3]) produces straight or wavy spore chains which are not characteristic of the grey streptomycetes causing scab. We did not find "rectus" variants among our *S. scabies* isolates. In our opinion the capacity to produce grey mature aerial mycelium (spores en masse), spiral sporophores, smooth spores, and the ability to utilize all ISP diagnostic carbon sources are distinguishing diagnostic features of the species. On the other hand, the production of melanoid pigments seems to be a variable biochemical feature of *S. scabies* and it is not available for species differentiation. Pure M⁻ populations of *S. scabies* can colonize and attack potato tubers and cause scab lesions. GREGORY and VAISEY [11] have already shown that 12 tyrosinase-deficient mutants of *S. scabies* were virulent for potatoes in controlled greenhouse tests.

Serological relationships between the potato scab organisms and other non-pathogenic species of streptomycetes have been investigated by several workers [12, etc.]. Because the strains of the non-pathogenic species were selected without considering their taxonomic relationship to *S. scabies* and the strains of the latter have not been adequately identified diagnostically, the results are taxonomically hardly evaluable. Douglas and Garrard [13], e.g. reported that there was no clear-cut division into pathogenic and saprophytic groups on the basis of serology.

Streptomycetes belonging to different species occur frequently in scab lesions of potato tubers, among the hyphal filaments of *S. scabies*. Perhaps a certain type of beneficial interaction may exist between these organisms and

S. scabies. There is unfortunately, little information in the literature on their systematic position and biochemical properties. Our "flaviovirens"-isolates too, remain undetermined.

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DEVELOPMENT OF SPECIFIC CELLULAR IMMUNOREACTIVITY IN TYPHOID FEVER

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Development of cellular immunoreactivity to *Salmonella typhi* and *Salmonella paratyphi-A* was studied by the leukocyte migration inhibition test in 9 patients with typhoid fever and in 2 patients with paratyphoid fever. Cellular reactivity could be demonstrated from the first days of the disease in all the subjects. The most pronounced migration inhibition was observed during the febrile period. It is suggested that specific cellular reactivity may play a pathogenetic role in typhoid fever.

In a previous study we have observed a specific cellular reactivity to *Salmonella typhi* in typhoid patients during convalescence and six months later [1]. The present investigations were undertaken to determine the development of this reactivity during the acute phase of the disease.

Patients and methods

Eleven male patients between 24 and 54 years of age were investigated. Nine had typhoid fever and 2 had *Salmonella paratyphi-A* infection. All of them were sporadic cases. The diagnosis established on the basis of a positive Widal test. The pathogenic agent was isolated from the blood or faeces of 10 patients. Eight patients were treated with chloramphenicol, 2 with thrimethoprim + sulphamethoxazol and 1 with ampicillin. Fever ceased usually one week after the introduction of antibiotic therapy. The controls were 12 patients aged 21 to 64 years with the diagnoses: leptospirosis in 4; lymphocytic choriomeningitis and infectious mononucleosis in 1 case each. All control patients had fever at the time of the LMI test.

Cellular reactivity was tested by the leukocyte migration inhibition (LMI) test as described by SØBORG and BENDIXEN [2]. Antigens were heat-inactivated *S. typhi* and *S. paratyphi-A* suspensions from 24 hr agar cultures. *S. typhi* strain (antigenic structure: 9,12, Vi: d: -) was kindly supplied by the National Institute of Hygiene. *S. paratyphi-A* strains were freshly isolated from the patients under study. Antigens were used in a concentration of 5×10^7 cells per ml medium. Migration indices were calculated from the means of migration areas in triplicate culture chambers. According to the statistical evaluation of our previous results, by Student's *t* method the LMI test was considered positive if the migration index was less than 0.8.

In typhoid and paratyphoid patients LMI tests were done several times at 1 to 2 week intervals. Controls were tested once during the febrile period.

Results

Table I shows the MI indices of typhoid and paratyphoid patients. Migration inhibition could be demonstrated in all patients. In 7 patients the test was positive on each occasion, the lowest indices were found mostly at the time of the clinical symptoms.

Table I

Leukocyte migration inhibition indices of typhoid (paratyphoid) patients during the disease

Patient	Weeks after onset of disease											
	1	2	3	4	5	6	7	8	9	10	11	12
A. K.	0.57	0.72	0.71						0.73			
B. D.			0.92	0.72			0.60	0.55	0.50	0.59		0.64
M. B.*		0.59	0.41	0.57		0.80						0.89
V. M.		0.64		0.59		0.77			0.65			
M. I.		0.54		0.78	0.77							
I. J.				0.68		0.62		0.65				0.69
P. J.		0.55		0.69		0.73	0.93					
M. B.				0.75			0.89	0.85		0.88		
K. S.					0.76		0.68	0.62		0.58		
B. N.*				0.60	0.61		0.62				0.64	
S. J.			0.50	0.66								

Figures in italics mean that the test was made in the period of symptoms

* Paratyphoid patients (*S. paratyphi-A* infection)

Figure 1 shows the LMI indices of 8 patients tested both during and after the acute phase of the disease. (If more than one test was done during the febrile period, the lowest MI-value is presented in the figure.) It is seen

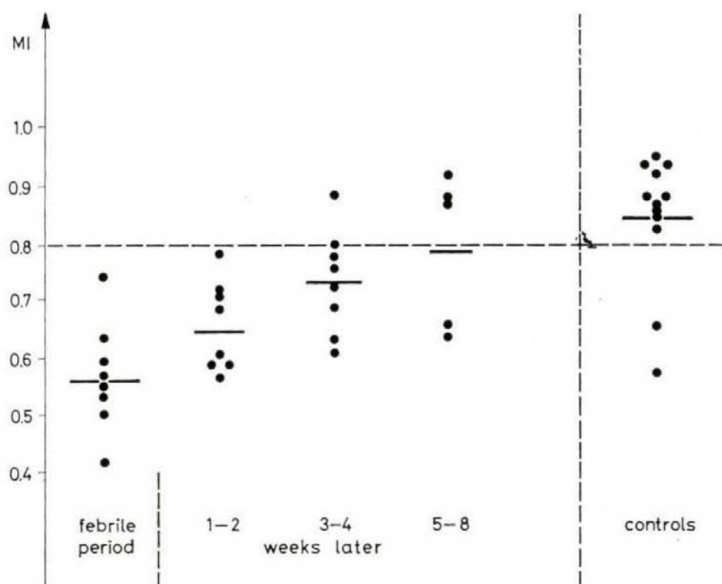


Fig. 1. MI-values in typhoid (paratyphoid) patients and controls

that specific cellular reactivity diminished with time, the lowest mean values were found during the febrile period. These findings raised doubt as to the specificity of the LMI test. This was why febrile patients served as controls. MI-values of these patients are presented in Fig. 1. Inhibition was significant in 2 cases, one of leptospirosis and one of pneumonia. The mean of the controls differed significantly from that of the febrile typhoid patients, as calculated by the *t*-test ($p < 0.001$). Moreover, the mean for the controls was significantly higher than that for the typhoid patients even 1 to 2 or 3 to 4 weeks after the period of fever ($p < 0.001$ and $p = 0.001-0.002$, respectively). As mentioned above one of the LMI-positive controls was a patient with leptospirosis. The diagnosis was verified by a rise in the specific antibody titre. In this case the Widal reaction, too, was positive, its titre, however, did not change during the course of the disease.

As to the possible relation between clinical symptoms and specific cellular reactivity, two patients merit attention.

A. K. was treated with prednisolone for a progressively developing typhoid state, hepato-splenomegaly, and raised SGOT and SGPT in spite of antibiotic treatment. Steroid treatment resulted in a rapid improvement: the fever ceased, the hepato-splenomegaly regressed and consciousness cleared up. Before steroid treatment the MI index was 0.57; it rose to 0.72 during steroid therapy and persisted at that level.

B. D. was admitted with a history of fever lasting three weeks. Immediately after admission, still febrile, the MI-value was 0.92. After chloramphenicol treatment the temperature became normal and the MI-values decreased progressively. In the 7th week of the disease migration indices of 0.6 and 0.55 were found and a relapse with fever appeared. After ampicillin treatment the patient again became afebrile. The improvement was accompanied by slowly growing MI indices. The patient was discharged symptom-free, but he remained an *S. typhi* carrier.

Discussion

The present investigations have confirmed previous observations according to which typhoid patients developed a cellular immunoreactivity to *S. typhi* [1, 3, 4]. This reactivity could usually be demonstrated during the early period of the disease and reached the peak in the period of clinical symptoms, similarly as in the patients of BALAKRISHNA *et al.* [3]. In our material, specific cellular reactivity decreased during convalescence. The decrease, however, may be transitory and the specific cellular reactivity is likely to reappear later on, as LMI-positivity in typhoid patients many months after recovery [1]. Obviously, it is never known whether a transitory disappearance of specific cellular reactivity is a characteristic feature of typhoid fever or is due to the

therapy, though BALAKRISHNA *et al.* [3] observed no immunosuppressive effect of chloramphenicol as tested by LMI.

The question of primary importance remains whether the specific cellular reactivity to *S. typhi* or *S. paratyphi-A* has any role in the pathogenesis of typhoid or paratyphoid fever, respectively, as it was suggested by HÖRING [5-7]. Although a simultaneous occurrence of different phenomena means no unconditional causality, the following observations suggest that symptoms of the disease may at least partly be due to the specific cellular immunoreactivity. In the majority of cases the lowest MI-values were found at the time of clinical symptoms. Furthermore, corticosteroid treatment of the most severe, antibiotic-resistant case was followed by a rapid improvement of the condition along with the decrease of specific cellular reactivity. Finally, a relapse was observed in that single case in which the specific cellular reactivity developed slowly and the time of its appearance coincided with the relapse.

Although the above observations speak for the role of the specific cellular reactivity in the pathogenesis of typhoid (paratyphoid) fever, we think that LMI tests are not likely to give more information in this respect.

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DEVELOPMENT OF YEAST POPULATIONS IN MIXED CULTURES

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An equation has been worked out to describe the invasion of a yeast culture by a contaminant or a mutant. The equation applies to a culture of two strains with different growth rates. It is shown that a small difference in growth rates can cause a quick selection.

Continuous fermentation with yeast and bacteria, either in sterile medium (Simba process), or in non-sterile medium (N-alkane degradation), has wide industrial applications.

Prolonged cultures in media, sometimes very different in composition from the usual substrates may give rise to an evolution of populations.

This work, which is a first approach to the phenomenon, is a study of the invasion kinetics of a culture of *Saccharomyces cerevisiae* by a contaminant and also by a mutant.

Materials and methods

Biological material. Three strains of yeast were used: *Saccharomyces cerevisiae* Hansen, strain 4, a diploid strain (GALZY [1]) obtained in our laboratory. This strain gives rough colonies on solid media not containing hexose sugars. *Saccharomyces cerevisiae* Hansen, strain 183. This strain is isogenic from the preceding one, except for a couple of allelic genes. The mutation corresponding to the gene PLi5 confers the property of giving smooth colony formation on solid media, when the carbon source is not a hexose. *Kluyveromyces fragilis* Van der Walt. A clone selected in our laboratory from the CBS strain K 1555.

Methods of culture. The cultures were made in 100 ml Erlenmeyer flasks, filled to a tenth of their volume with G medium [1]. This medium contained 1% of lactic acid buffered to pH 4.5. The cultures were aerated by mechanical agitation (80 oscillations per min, amplitude 8 cm) at a temperature of 25 °C. The components of G medium were calculated in such a way that the limiting nutrient of growth was lactic acid. Under the experimental conditions, there were 6 to 7 generations for each of the three strains, before the stationary phase of growth. The flasks were regularly replicated after two to three generations. Then, the yeasts were maintained strictly in the exponential phase of growth during the experiment.

Lactic acid (analytical) was made by Mallinckrodt.

The population was controlled by counting and by turbidity measurements with a Klett Summerson photocolorimeter (filter No. 42).

Methods for recognizing the strains. A dilution of the culture is spread out on solid medium C [1] (Bacto Yeast extract Difco 0.5%, lactic acid 1%), in Petri dishes, in such a way as to obtain 100 to 300 colonies per dish. The plates are examined after 4 to 6 days, when the strain K 1555 gives typical, large, rough colonies. Strain 183 gives smaller lens-shaped perfectly smooth colonies. Strain 4 gives rough colonies, smaller than the colonies of K 1555 but perfectly distinguishable from those of 183.

Results

I. Invasion of a culture by a contaminant

(A) *Theoretical aspects of the invasion of a culture.* We shall limit ourselves to the study of cultures of strains whose population growth follows MONOD's law [2], thus we shall assume that the experimental conditions are such that the cultures are always in the exponential phase of growth. We shall suppose that the strains in mixed culture do not interfere with each other, and that each strain behaves as if it were alone in the medium.

We shall call X the population of the first strain; μ_1 its growth rate; X_0 its population at the beginning of the experiment; Y the population of the invading strain; μ_2 its growth rate; Y_0 its population at the beginning of the experiment; T time (hr); T_0 time at the beginning of the experiment. Let us say $Y \ll X$ and $\mu_2 < \mu_1$. The proportion (P) of the contaminant (Y) is described as

$$P = \frac{Y}{X}$$

and at $T = T_0$

$$P_0 = \frac{Y_0}{X_0}$$

On the basis of assumptions referred to at the beginning of this section, we obtain

$$\frac{dX}{dT} = \mu_1 X$$

$$\frac{dY}{dT} = \mu_2 Y$$

thus

$$X = X_0 e^{\mu_1(T-T_0)}$$

$$Y = Y_0 e^{\mu_2(T-T_0)}$$

consequently

$$P = \frac{Y}{X} = \frac{Y_0}{X_0} e^{(\mu_2 - \mu_1)(T - T_0)} \quad (1)$$

This equation is only valid in the case of cultures maintained in the exponential phase of growth by successive sub-cultures. Figure 1 shows the development of the proportion of the contaminant as a function of time for two values of $\mu_2 - \mu_1$.

Let us say T_{10} is the time required for the proportion of the contaminant to increase tenfold. From equation (1), therefore, we deduce

$$\ln 10 = (\mu_2 - \mu_1) T_{10}$$

thus

$$T_{10} = \frac{\ln 10}{\mu_2 - \mu_1} \quad (2)$$

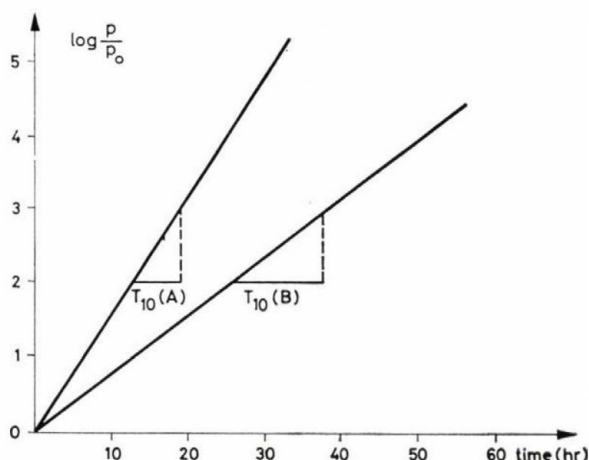


Fig. 1. Evolution of the proportion of contaminants for two values for $\mu_2 - \mu_1$

Figure 2 gives the values $\frac{1}{T_{10}}$ as a function of the difference between growth rates.

(B) *Experimental results.* A culture of the yeast *S. cerevisiae* strain 183 of a smooth colony phenotype, is mixed with a culture of the strain K 1555. This strain has a rough colonial phenotype.

At the time of mixing, the two strains are in the exponential phase of growth. Inoculations are made so as to have an initial rough colony frequency of 1% in the mixture.

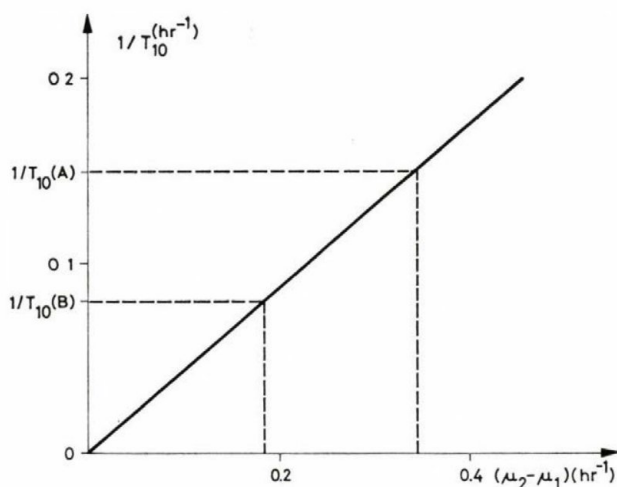


Fig. 2. Values of $1/T_{10}$ in terms of $\mu_2 - \mu_1$

The relative proportion of rough colonies and smooth colonies is monitored during the time necessary for the total elimination of the smooth colonies of *S. cerevisiae* strain 183. That means that the experiments were stopped when no smooth colony was observed in 1000 colonies, in two consecutive spreadings.

Figure 3 shows the evolution of the proportion of K 1555 in a mixed culture. The kinetics of invasion follows an exponential law. The invasion law is analogous to the theoretical law of invasion. The experimental T 10 observed in independant experiments varies from 6 hr 15 min to 9 hr 45 min.

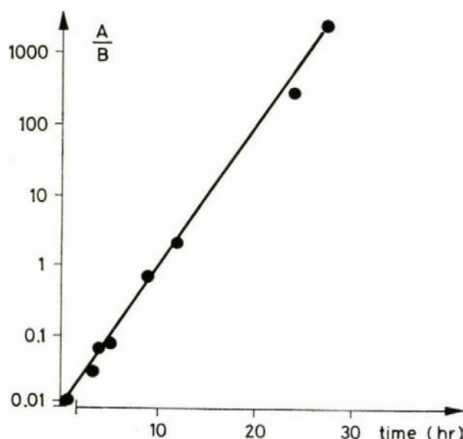


Fig. 3. Invasion of a culture of *S. cerevisiae* strain 183 by strain CBS K 1555 of *K. fragilis*;
y axis: ratio of contaminant A to initial strain B, $P = \frac{A}{B}$; x axis: time (hr)

The Napierian growth rates ($\mu_N = \ln 2/\text{generation time}$) were measured in discontinuous cultures, in experimental conditions strictly identical with those of the selection experiments. The growth rate of strain 183 is 0.099 hr^{-1} with a standard deviation of 0.012 hr^{-1} (number of determinations $N = 9$). The growth rate of strain K 1555 is 0.431 hr^{-1} with a standard deviation of 0.114 hr^{-1} (number of independant determination $N = 8$).

Equation (1) allows to calculate the theoretical T 10. This value is 7 hr, when calculated from the mean values of the μ_N given above.

Consequently, the theoretical T 10 and the experimental T 10 are the same, taking into account the biological fluctuations and experimental errors. The theoretical law is thus verified.

II. Invasion of a culture by a mutant

(A) *Theoretical aspects.* When a mutation occurs in a culture, the individuals that bear it invade the culture if the mutation increases their growth rate.

Equation (1) applies to such a case. Mutations are, however, generally recurrent and the effect of the mutation rate α must therefore be taken in

account in a study of the phenomenon of selection. If y is the population of the mutant and α the mutation ratio, $\frac{dy}{T} - \mu_2 y = \alpha \mu_1 \frac{dX}{dT}$

Taking into account equation (1)

$$(2) \quad P = P_0 e^{(\mu_2 - \mu_1 + \alpha)(T - T_0)} - \alpha \frac{\mu_1}{\mu_2 - \mu_1 + \alpha} [e^{(\mu_2 - \mu_1 + \alpha)(T - T_0)} - 1]$$

α is very small and the second member of equation (2) is very small in relation to the first term. Thus it can be neglected as much as it is a spontaneous mutation rate.

With a high mutation ratio, for instance in the presence of a mutagenic agent, the case would not be the same.

In a study of the action of acriflavine on yeast, EPHRUSSI *et al.* [3] have pointed out that this ratio becomes preponderant during the induction of the transformation by acriflavine of the large strain into small strains. In the following studies we may neglect the α parameter.

(B) *Experimental results.* Smooth colony mutants are obtained by selection during prolonged culturing on media with lactic acid as the only carbon source [1]. Figure 4 shows the invasion of a culture of strain 4 by the smooth colony strain 183 in these particular conditions. Invasion of strain 4 by strain 183 occurs with a T_{10} of about 180 hr.

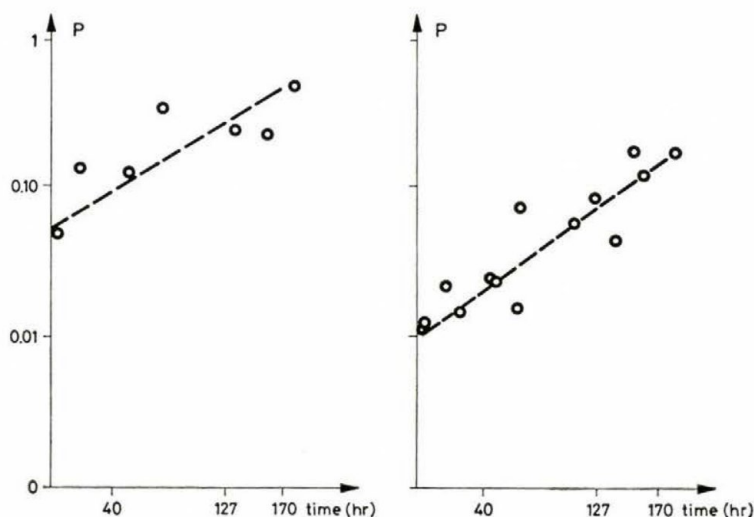


Fig. 4. Invasion of a culture of *S. cerevisiae* strain 4 by strain 183 of *S. cerevisiae* (two independent experiments); y axis: ratio of contaminant A to initial strain B, $P = \frac{A}{B}$; x axis: time (hr)

Measurement of the growth rate of the two strains was done by nephelometry. There was no significant difference between the two strains. We found $\mu_1 = 0.102 \text{ hr}^{-1}$ ($\sigma = 0.016$, $N = 9$) with strain 4 and $\mu_2 = 0.099 \text{ hr}^{-1}$ ($\sigma = 0.012$, $N = 9$) with strain 183. The growth rate margin required red for explaining a T 10 of 200 hr is $\Delta\mu = 0.0115$. It is thus of the order of magnitude of the normal errors of estimation. Accuracy of measurements does not allow to point out a difference in the growth rates which is nevertheless sufficient to give a selection.

This last example shows that a small difference in growth rate can be responsible for the fast replacement of a strain by another. This invasion is a better way to define the selective advantage of a strain in a given medium than to measure its growth rate.

Discussion

Some authors have studied mutant selection in microbial strains [4, 5], others have studied steady state mixed cultures with the practical aim of regulating fermentors [6, 7]. Our long-term aim will be to establish a relationship between the genetic and physiological characteristics of microorganisms and the phenomenon of selection in mixed cultures.

In the present investigation we have studied the simple case of a yeast culture invaded by another yeast. We had to make a certain number of very restrictive assumptions. Equation (1) is not valid when an antibiosis exists between the two strains, or when the competition for a nutrient modifies their growth rate.

Another element which should be taken into consideration is the fact that the growth rate of a strain, on a given medium, is subject to notable fluctuations [8, 9]. Nevertheless, the equation is valid for the cases studied. On the other hand, it is not possible to predict the rate of invasion when mixed strains have similar growth rates. In such case, to forecast the behaviour of two mixed strains, a selection assay seems to be necessary.

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QUANTITATIVE DETERMINATION OF STAPHYLOCOCCAL B-TYPE ENTEROTOXIN WITH LAURELL'S ELECTROIMMUNE DIFFUSION METHOD

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The quantitative determination of B-type enterotoxin produced by *Staphylococcus aureus* is described. Pretreating the toxin with formaldehyde, the B-type enterotoxin could be demonstrated rapidly with appropriate sensitivity by LAURELL's electroimmune diffusion method. Four to eight hours were required for the examination and 0.25 µg/ml was the smallest demonstrable quantity of the toxin.

According to present knowledge, the enterotoxin is the principal aetiological factor in staphylococcal food poisoning. Thus the demonstration of toxin production is of great importance in revealing the origin of such poisonings. It serves for protection against toxin producing strains, i.e. for prevention of food poisonings. Several methods have been described for the demonstration of enterotoxins, among them the widely used agar-gel diffusion method [1, 2], haemagglutination inhibition and passive haemagglutination [3, 4], immune fluorescence [5], radioimmune method [6, 7], electroimmune diffusion [8] and the reverse immune osmophoresis methods [9].

The aim of the present work was to introduce, in possession of a specific anti-enterotoxin, a quick and sensitive method for the quantitative demonstration of B-type enterotoxin. LAURELL's electroimmune diffusion method was found to be the most suitable for the purpose. As up to now the method has only been used for A-type enterotoxins, experiments were carried out to apply it for the determination of B-type enterotoxin.

Materials and methods

Buffer. Barbitol sodium-sodium acetate buffer of $\mu = 0.1$ and 0.05 ionic strength and pH 8.6 was used [10].

Antigen. The crude B-type enterotoxin was kindly provided by Professor M. S. BERGDOLL (Food Research Institute, USA). The enterotoxin content of the lyophilized material was determined. The crude material was dissolved in 1 ml pH 8.6 buffer of $\mu = 0.05$ ionic strength and stored at -20°C until use.

Antiserum. Preparation of immune serum against B-type enterotoxin and its specificity control have been described earlier [11].

Pretreatment of the antigen (B-type enterotoxin) for electroimmune diffusion. Formaldehyde solution of 36% concentration (Chemapol, Czechoslovakia) was added to the buffer (pH 8.6, ionic strength $\mu = 0.05$) until 0.6% end concentration had been reached. The crude

enterotoxin was diluted in the buffer at a rate to produce 16 $\mu\text{g}/\text{ml}$ B-type enterotoxin concentration. Subsequently, the two solutions were mixed in identical proportion; the solution obtained contained 0.3% formaldehyde and 8 $\mu\text{g}/\text{ml}$ of B-type enterotoxin. In sealed glass tubes the solution was incubated in a 45 °C water bath for 1 hr.

Preparation of the specific immune serum containing agarose-gel plate. Agarose solution (Reanal) of 0.2% concentration was layered on carefully cleaned slides, immediately poured off and the slides were dried at 60 °C for 5–10 min. The agarose-immune serum was prepared in the following way. As in earlier studies a 1:60 mixture of immune serum and agarose was found the most suitable, one part of specific immune serum was added to 59 parts of hot (50 °C) 1% agarose solution in barbital sodium-sodium acetate buffer pH 8.6 ionic strength $\mu = 0.05$, and the solutions were thoroughly mixed. Of the warm mixture 2 ml was placed on 2 slides covered with 0.2% agarose. Three wells 3 mm in diameter, were cut in the solid plates approximately 25 mm from the edge of the slide and the disks were removed by vacuum. The slides were kept in a moist chamber until used.

Electrophoresis. The agarose plates were placed in the slide holder of the tank fitted with a cooling device. The antigen wells faced the cathode. Barbital sodium-sodium acetate buffer of $\mu = 0.1$ ionic strength and pH 8.6 was placed into the tank and Whatman 3MM filter paper served for the connection between buffer and agarose plate. The antigen reservoir was filled with 0.007 ml formaldehyde treated B-type enterotoxin at concentrations of 8, 4, 2, 1, 0.5 and 0.25 $\mu\text{g}/\text{ml}$. Electrophoresis was carried out for 1–5 hr at 5 mA/plate current intensity and 100 V. Tap water was used for cooling.

Detection of precipitation peaks. After electrophoresis the agarose plates were placed into physiological saline. Proteins which did not take part in formation of the precipitate dissolved in the saline; the procedure took about 1.5 hr during which time the saline was changed several times. Then the plates were put in distilled water to eliminate the salt from the agarose, and then into 96% ethyl alcohol for 10–20 min for preventing the agarose-gel to split during the drying procedure. Drying was performed either in a drying chamber at 60 °C or with a hair-drier. The plates were stained with 0.1% amido black dissolved in 7% acetic acid for 10–15 min. The excess stain was removed with 7% acetic acid and the plates were dried again.

Immune electrophoresis. Immune electrophoresis was carried out according to SCHEIDEGGER [12] in 1% agarose-gel dissolved in the above described buffer.

Results

The agarose plate with 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 $\mu\text{g}/\text{ml}$ toxin is presented in Fig. 1.

Migrating in the specific immune serum containing agarose, the formaldehyde treated B-type enterotoxin formed precipitation peaks varying in height. The height of the peaks depended on the quantity of enterotoxin. After a 5 hr electrophoresis, the correlation between the quantity of enterotoxin ($\mu\text{g}/\text{ml}$) and the height of the precipitation peaks (mm) is presented by a calibration curve in Fig. 2. There was a linear relationship between the quantity of enterotoxin and the height of the precipitation peaks (Fig. 2). The calibration curve allowed to determine the quantity of B-type enterotoxin in the filtrate of the *S. aureus* strain on the basis of the height of the precipitation peaks.

Discussion

GASPER *et al.* [8] applied the modified electroimmune diffusion technique of LAURELL for the demonstration of A-type enterotoxin [13, 14]. Attempts were made to apply the method also for the demonstration of B-type enterotoxin, but it failed to give reliable results. The failure might have been due to

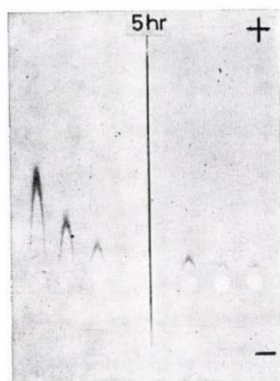


Fig. 1. Quantitative determination of B-type enterotoxin with Laurell's electroimmune diffusion. Enterotoxin quantity (from left to right): 8, 4, 2, 1, 0.5 and 0.25 $\mu\text{g/ml}$. Dilution of the specific anti-enterotoxin immune serum 1: 60. Duration of electrophoresis, 5 hr

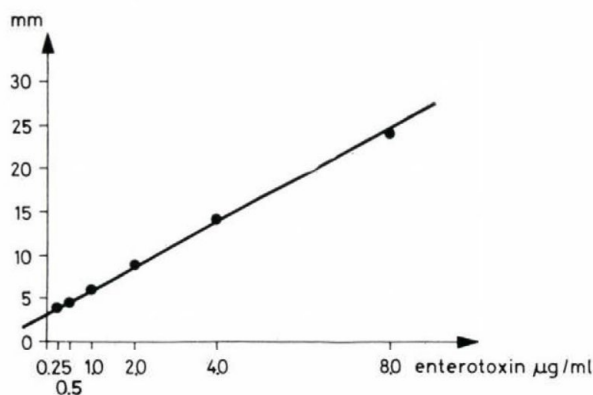


Fig. 2. Correlation between the quantity of enterotoxin and the height of the precipitation peaks after 5 hr electrophoresis. Dilution of the specific anti-enterotoxin immune serum, 1: 60

the fact that while the isoelectric point of A-type enterotoxin is at pH 7.3, consequently its molecule disposes of a certain excess charge in pH 8.6 agarose-gel, the charge of B-type enterotoxin is zero under the same circumstances, its isoelectric point being at pH 8.6. Thus, in agarose-gel of pH 8.6 the A-type enterotoxin migrates towards the anode, whereas the B-type toxin fails to change its position.

In the course of purification of B-type enterotoxin and the preparation of anti-enterotoxin, the enterotoxin being transformed by formaldehyde treatment showed other migration characteristics in electrophoresis than did the untreated enterotoxin. It was assumed therefore, that the formaldehyde treated B-type enterotoxin could be made suitable for Laurell's electroimmune-diffusion. Examinations performed with this purpose are presented in Fig. 3.

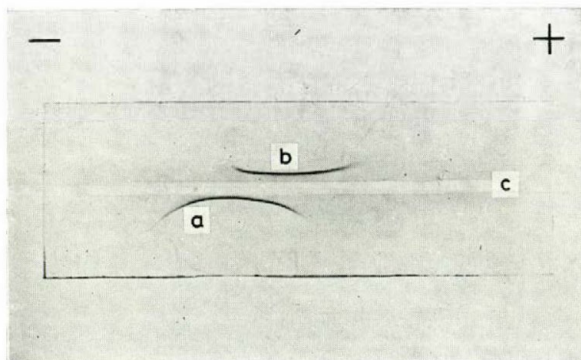


Fig. 3. Formaldehyde treated (formaldehyde 0.3%, in 45 °C water bath for 1 hr) and native B-type enterotoxin compared in agarose-gel immune electrophoresis, a = precipitation line of native B-type enterotoxin; b = precipitation line of formaldehyde treated B-type enterotoxin; c = reservoir for the specific anti-enterotoxin immune serum

Figure 3 shows that after electrophoresis in agarose of pH 8.6, the formaldehyde treated enterotoxin migrated towards the positive electrode while the untreated enterotoxin formed a precipitation line around its well, indicating that the native enterotoxin did not change its position during electrophoresis. Formaldehyde treatment changed the charge of the toxin molecule transformed into toxoid, making thus possible the rapid demonstration of the presence and quantity of the toxin.

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THE HETEROGENEITY OF FUSARIUM CULMORUM NUCLEAR DNA

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Some characteristics obtained by means of the thermal denaturation technique of the nuclear DNA isolated from the filamentous fungus *Fusarium culmorum* W. G. Smith Sacc. are described. *F. culmorum* nuclear DNA has a heterogeneous base composition as shown by the width of thermal transition (14.8 °C), and a polyphasic thermal denaturation curve. When percent hyperchromicity was plotted against temperature on normal probability graph paper, nuclear DNA exhibited the presence of three distinct hyperchromic fractions, i.e. an adenine plus thymine (A+T) rich fraction, a main band, and a guanine plus cytosine (G+C) rich fraction. These three fractions are interpreted to reflect the presence of a non-Gaussian distribution of bases and thus an intramolecular heterogeneity within the nuclear DNA. The differential form of the thermal denaturation profile of *F. culmorum* nuclear DNA seemed to indicate that the discontinuous melting of DNA suggests the presence of several populations of DNA molecules.

The optical melting profile of natural DNAs points to an intramolecular heterogeneity. It is well-known that eukaryotic DNA is heterogeneous in base composition. This heterogeneity arises from a limited number of DNA components different in base composition or from a continuous distribution of sequences different in base composition. A linear relationship between melting temperature (T_m , midpoint of optical density rise during denaturation) and mole per cent G+C content of DNA offer a convenient method for DNA characterization [1]. The transition width of the melting profile has been used as a rough estimate of the heterogeneity of nucleotide distribution (intramolecular heterogeneity) in a single DNA species [2, 3]. If the DNA melting profile is unusually broad and, in some cases, even polyphasic, then the DNA has a heterogeneous base composition. Polyphasic denaturation profiles due to intramolecular heterogeneity have been reported for several fungal DNAs [4–8]. The DNA of *Fusarium* has scarcely been studied.

In the present work, characterization of the nuclear DNA (nDNA) of *F. culmorum* W. G. Smith Sacc. was achieved by the thermal denaturation technique. nDNA was investigated with respect to thermal melting (T_m) values and calculated base composition (mole per cent G+C). Intramolecular heterogeneity was studied using normal probability plots of the thermal denaturation profile data. Different DNA molecule populations were revealed by the differential form of the thermal denaturation profile.

Materials and methods

Organism and cultivation. *F. culmorum* strain F-6 isolated from wheat stalk was maintained in oil culture in the refrigerator. Cultivation of the fungus has been described elsewhere [9].

Isolation and purification of DNA. DNA extraction and purification have been described earlier [9]. The washed mycelium was lyophilized and powdered in a Mortar Grinder (Model RMO, Retsch KG). The lyophilized and powdered material was used for DNA purification. One g of dry hydroxiapatite (DNA grade Bio-Gel HTP, Bio-Rad) was used for isolation of DNA from 1 g mycelium powder. Hydroxiapatite (HA) was suspended in 0.01 M sodium phosphate buffer (SPB, equimolar solutions of NaH_2PO_4 and Na_2HPO_4 , pH 6.8) and heated to the boil. It was equilibrated with MUP (8 M urea–0.24 M SPB, pH 6.8) and used for DNA purification as described previously [9]. The nDNA was separated from the mycelial DNAs by four successive adsorption of DNA to HA and elution with 0.28 M SPB [10, 11]. Purity of DNA preparations was monitored by optical spectra; $A_{260}/A_{280} = 1.88$ to 1.90; $A_{260}/A_{280} = 2.28$ to 2.30.

Determination of molecular weight of nDNA. The molecular weight of unsheared DNA samples was determined using the equation proposed by CROTHERS and ZIMM [12]: $0.665 \log M_w = 2.863 + \log ((\eta) + 5)$. Intrinsic viscosities were determined in an Ostwald type 4 viscosimeter ($K_{20} = 0.006763$). The concentration of DNA in $0.1 \times \text{SSC}$ (0.015 M NaCl plus 0.0015 M sodium citrate, pH 7.0) was 100 $\mu\text{g}/\text{ml}$ ($A_{260} = 2.0$). Concentration effects were corrected by the expression: $\eta_{sp}/C = (\eta) + k'(\eta)^2C$ with a value k' of 0.5 as proposed by EIGNER *et al.* [13]. The molecular weight of *F. culmorum* unsheared nDNA was 3.45×10^6 daltons.

Sample preparation for thermal denaturation. The unsheared DNA solutions were dialysed against five changes of $0.1 \times \text{SSC}$ solution to avoid differences in solvent conditions. They were used as stock solutions.

Thermal denaturation technique. Thermal denaturation curves were automatically and continuously recorded by an Unicam SP 1750 Ultraviolet Spectrophotometer (Pye Unicam Ltd., England) equipped with an SP 876 Series 2 Temperature Programme Controller, heated cell block and an X–Y recorder (PM 8041, Philips). An unsheared DNA sample in $0.1 \times \text{SSC}$ was first passed through a Metrical GA-6 (Gelman) filter to remove dust particles and then degassed under partial vacuum for 10 min. Absorbance (A_{260}) of the DNA solution was adjusted to around 0.7 at the beginning of heating. The blank ($0.1 \times \text{SSC}$) was substituted for guanosine dissolved in $0.1 \times \text{SSC}$ to an absorbance equal to that of the sample. Full-scale deflection of the recorder pen on Y-axis was adjusted to an absorbance (A_{260}) window 0.000–0.200 (Recorder Range: 0.2 A) in every recording. Noise levels were reduced by large (1.0 mm) slit widths together with increased DNA concentrations [14]. The DNA sample and the blank were placed in UV grade silica cells (Pye Unicam Ltd., 1.3 ml, 5 mm path length). The cuvettes were filled so that the air space was less than 10% of the fluid volume [1]. Evaporation was prevented with a teflon stopper to less than 0.5% per hr. The temperature of the Electrically Heated Cell Block (Pye Unicam Ltd.) was raised to 30 °C, then a programmed temperature rise of 0.25 °C/min was provided by a Unicam Temperature Programme Controller. Heating was continued until complete denaturation. Recorded melting curves (absorbance values) were corrected for thermal expansion of water [1], normalized to the total hyperchromicity and transformed into a differential form by numerical differentiation. For numerical calculations, every Y-value (absorbance) was read from the chart of a crude thermal denaturation profile on every scaled points of the X-axis (temperature). The temperature difference between two sampled points was 0.4 °C. The base composition of DNA samples was calculated from T_m values, the temperature corresponding to 50% of the hyperchromic rise measured at 260 nm [1]. The mole percent of polymerized guanine plus cytosine (mole percent G+C) of DNA in $0.1 \times \text{SSC}$ was calculated from the empirical equation: mole per cent G+C = $(T_m - 53.9) \times 2.44$ [1]. The base compositional distribution (intramolecular heterogeneity) within the DNA molecules of the sample was determined by re-plotting the thermal denaturation profile data onto normal probability graph paper and the temperature differences for 15.8% and 84.1% of the total increase in absorption (transition interval, ΔT). If the denaturation profile could be replaced by a Gaussian one [2] it would be equal to the standard deviation (2σ). As, however, the denaturation profile is mostly asymmetrical, the approximation is inaccurate. Nevertheless, the values for ΔT can still be taken as a rough measure of the heterogeneity of DNA. The ΔT value was determined on the normal probability plot [15]. The presence of "melting fractions" of DNA molecules was revealed by construction of the differential form of the thermal denaturation profile [16].

Results

Thermal denaturation profile of *F. culmorum* nDNA. Figure 1 shows the melting profile of *F. culmorum* nDNA. The T_m of nDNA is 71.2 ± 0.1 °C (variation observed in 10 experiments). According to the relation for the $0.1 \times \text{SSC}$ solvent [1], the base composition of nDNA is 42.2% of G+C. The hyperchromicity is usually between 27–33%. The ΔT for *F. culmorum* nDNA is 14.8 °C (36.1% of G+C): from 61.2 °C to 76.0 °C (17.8 to 53.9% of G+C). The thermal denaturation profile of *F. culmorum* nDNA is not true of a sigmoidal shape; it shows an asymmetrical curve skewed toward the G+C rich region of the profile, an irregular, polyphasic curve (Fig. 1).

Normal probability plot of the thermal denaturation profile of *F. culmorum* nDNA. Figure 2 shows the normal probability plot of the thermal denaturation profile data of *F. culmorum* nDNA. When the nDNA data were re-plotted onto normal probability graph paper, a single straight line could not be drawn. Consequently, it appears that though *F. culmorum* nDNA is not Gaussian in base distribution but contains regions of non-random base pairs. On a normal probability plot the thermal denaturation profile of *F. culmorum* nDNA was found to be triphasic, indicative of the presence of 3 nDNA components. These three distinct hyperchromic fractions or slopes *A*, *B*, *C* (Fig. 2) are thought to reflect the presence of three different concentrations or families of nitrogenous bases in nDNA [17]. Slope *A* (low temperature denaturing fraction) melts first and presumably represents that portion of total increase

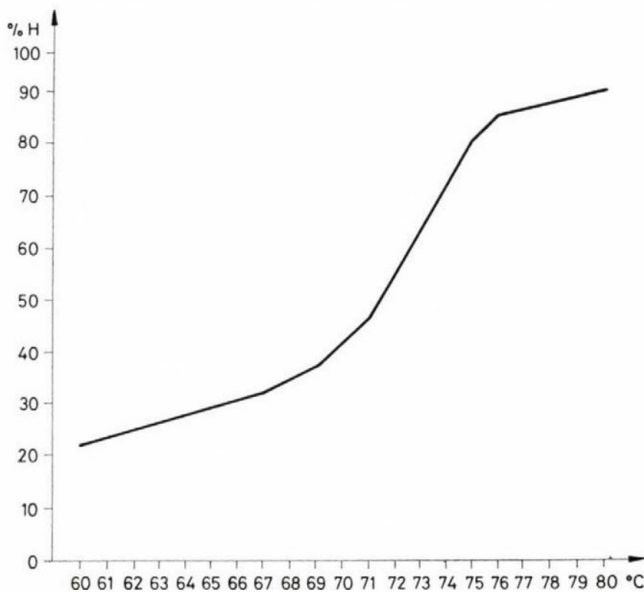


Fig. 1. Thermal denaturation profile of *F. culmorum* nDNA at 260 nm. % H = percent of total hyperchromicity

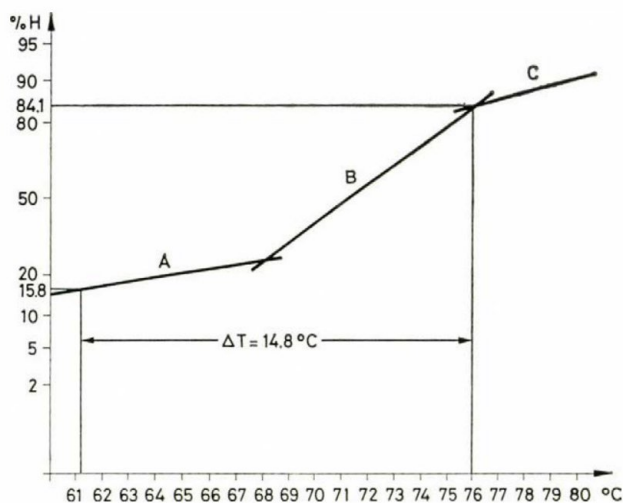


Fig. 2. Normal probability plot of thermal denaturation profile data of *F. culmorum* nDNA at 260 nm. % H = percent of total hyperchromicity

in hyperchromicity which is the adenine plus thymine enriched fraction. The mean percent of slope *A* represented by nDNA is 36.3. Slope *B* (medium temperature denaturing fraction) represents the main band DNA and the largest portion of the total hyperchromicity. The part of slope *B* represented by nDNA is 45.4%. Slope *C* (high temperature denaturing fraction) represents that portion of the total hyperchromicity which is the guanine plus cytosine enriched fraction. The percent of slope *C* represented by nDNA is 18.1. The *A*, *B*, *C* fractions have T_m values and moles per cent G+C of approximately 64°C (24.6), 72°C (44.1) and 78°C (58.8), respectively. Table I summarizes the thermal denaturation profile data of *F. culmorum* nDNA on a normal probability plot.

Table I

Thermal denaturation profile data on a normal probability plot of *F. culmorum* nDNA

Fraction	Percent	T_m in °C	Moles% G+C
<i>A</i>	36.3	64.0	24.6
<i>B</i>	45.4	72.0	44.1
<i>C</i>	18.1	78.0	58.8

Differential form of the thermal denaturation profile of F. culmorum nDNA. Figure 3 shows the differential form of the melting curve of *F. culmorum* nDNA. The differential curve shows 13 peaks; these peaks are qualita-

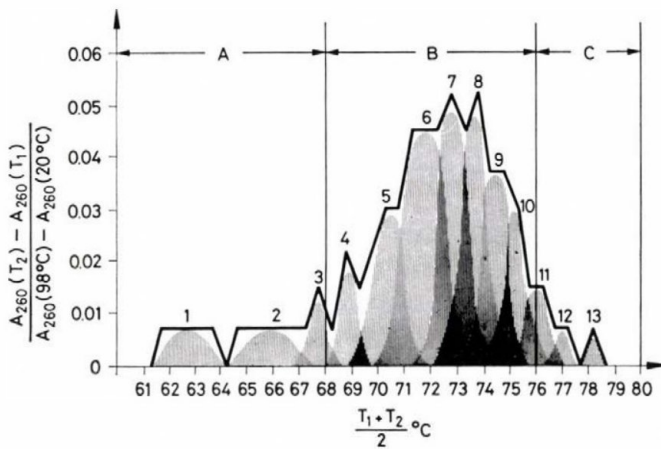


Fig. 3. Differential form of the thermal denaturation profile of *F. culmorum* nDNA. T_1 and T_2 are two sequential temperatures differing by 0.4 °C. The lined areas in the profile are the assumed melting components of nDNA

tively repetitive (according to 10 experiments). Comparing the normal probability plot and the differential form of the thermal denaturation profile of nDNA, it is apparent that the A, B, C fractions involve several subfractions. Fraction A contains two large subfractions and a smaller one; fraction B seven subfractions while fraction C contains three subfractions. Table II gives some of their parameters.

Table II

Thermal denaturation profile data on a differential plot of *F. culmorum* nDNA

Peak	T in °C*	Moles% G+C
1	62.7	21.4
2	65.9	29.2
3	67.7	33.6
4	68.7	36.1
5	70.5	40.5
6	71.7	43.4
7	72.8	46.1
8	73.7	48.3
9	74.5	50.2
10	75.3	52.2
11	76.0	53.9
12	77.0	56.3
13	78.2	59.2

* T in °C is the temperature for which a maximum peak is raised in the differential profile.

Discussion

The results suggest that *F. culmorum* nDNA is highly resolvable with the described techniques. *F. culmorum* nDNA shows a polyphasic thermal denaturation profile (Fig. 1); has an asymmetrical profile which is skewed to the G+C rich region.

The compositional heterogeneity of *F. culmorum* nDNA has been revealed by graphing the thermal denaturation profile of nDNA on a normal probability plot. This indicates that there are 3 components of nDNA in *F. culmorum* (Fig. 2, Table I). Such a heterogeneity has been reported in other fungal DNAs, too [6-8]. The present results suggest that a potentially meaningful way to characterize the intramolecular heterogeneity of DNA is by the use of thermal denaturation data plotted on normal probability graph paper [15, 17]. One way to describe the 3 components would be to interpret them as families or specific fractions of the molecular fragments of the DNA in each sample. Thus, each component could be defined as reflecting a region of intramolecular base compositional homogeneity. The differential form of the thermal denaturation profile of nDNA (Fig. 3) demonstrates, however, further subcomponents in the melting profile.

Another way to estimate overall base heterogeneity is to identify the transition interval (ΔT) between the thermal transitions in melting profile [2]. The larger the transition interval the greater the degree of heterogeneity in base composition. The great ΔT value (14.8 °C) of *F. culmorum* nDNA indicates a large heterogeneity in base composition. The transition interval is known to increase from phages to animals [18]. The ΔT values are directly comparable since only small variations of ΔT occur above a molecular weight of 1×10^6 daltons [3].

The differential form of the melting profile of *F. culmorum* nDNA deviates from a Gaussian distribution [3] and displays individual components (Fig. 3), which most likely indicate a multiphase denaturation within the DNA molecule. Presumably, the components of the differential plot represent different "melting families" of DNA segments similar in G+C content, but only further studies with high-resolution thermal denaturation technique [14, 19] and renaturation experiments will decide whether the peaks observed represent homogeneous families of DNA sequences, or else a mass of individual subfractions.

Careful analysis of the fine structure of DNA thermal denaturation profiles can contribute significant new informations about eukaryotic genomes. The genomes larger than a few hundred thousand base pairs point to "blocks" of DNA differing in their mean G+C content [20, 21]. Large "blocks" of DNA with distinctive base composition occur in both bacteria and eukaryotes [20, 22]. Each "block" should be composed of many DNA sequences having

nearly the same base composition and forming a family of sequences. HUGUET and JOUANIN [18] described a close relationship between taxonomical vicinity and thermal denaturation profiles of maize and wheat DNA. Some of the components with very high melting temperatures may be specific for the *Gramineae*, accounting for the exceptionally high contents of G+C in the DNA in this family [23]. The denaturation peaks should be representative of the species specificity of DNA. Therefore, thermal denaturation profiles may thus be of interest to taxonomy. In this respect it would be important to examine the DNAs of several fungal species of the same genus or family, determining the extent of homology between the separately melting components and correlating their occurrence with the karyotypes. A correlation between phylogenetic relations among mammals and the shape of the melting profiles of their nuclear DNAs was demonstrated by GUTTMANN *et al.* [24].

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DIFFERENTIATION OF RAPIDLY GROWING SCOTOCHROMOGENIC MYCOBACTERIA EMPLOYING THE ZYM API SYSTEM AS SUBSTRATUM

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Nineteen enzyme activities were investigated in 15 species of rapidly growing scotochromogenic mycobacteria, employing the ZYM API system as substrate. With this system, the time of identification is reduced from two weeks to four hours.

For the identification of rapidly growing scotochromogenic mycobacteria biochemical [1, 2] and immunological methods [3] and thin-layer chromatography [4] have been used. In contrast with these laborious and time consuming techniques, with the ZYM API system the time of identification is reduced to four hours.

The ZYM API system is a semi-quantitative micromethod designed for the research of enzyme activities. It can be applied to tissues, cells, biological fluids, microorganisms, washings, soil, oil, etc. [5–7]. It allows the systematic and rapid study of 19 enzymatic reactions in minute samples.

Material and methods

Bacterial strains. The fifty-four strains used in this study are listed in Table I. Some strains were identified in this laboratory by methods described previously [8].

Recommended procedure. The ZYM API system should be stored upon receipt at 2–8 °C in the refrigerator until used. The strip is composed of 20 microtubes, the bottom of which forms a support especially designed to contain the enzymatic substrate and buffer. The support allows contact between the enzyme and the generally insoluble substrate.

Preparation of strips. Dispense approximately 5 ml of tap water into the incubation tray to provide a humid atmosphere during incubation. A plastic squeeze bottle may be used.

Preparation of specimens. The strains are grown in Ogawa egg medium at 37 °C for 5 days. Dilute the specimen in a minimum volume of 2 ml of sterile distilled water or in another dilutant such as normal saline without any buffer. Prepare a suspension with a turbidity between MacFarland No. 5 and No. 6 standard.

Inoculation of the strip. With a Pasteur pipette, inoculate two drops (65 µl) of the specimen in each well of the strip.

Incubation of the strip. After inoculation, place the plastic lid on a tray and incubate it in the dark at 37 °C for 4 hr.

Reading the strip. After incubation, break an ampoule of the ZYM API reagent, add one drop of reagent A (Tris-hydroxymethyl aminoethane 250 g; hydrochloric acid 37% 110 ml; laurylsulphate, 100 g; distilled water to make 1000 ml, pH 7.6–7.8) and one drop of reagent B (Fast Blue BB-Sigma No. FO250, 3.5 g, and 2-methoxyethanol to make 1000 ml).

Let the colour develop for five minutes. If possible, put the strip under a powerful light source (1000 Watt bulb) for about 10 seconds with the bulb about 4 s above the well. This procedure eliminates any yellow colour appearing in the well due to the excess Fast Blue

which has not reacted. After light exposure, negative reactions become colourless. Placing the strip in daylight for a few minutes will produce comparable results.

Recording the reaction. A value ranging from 0 to 5 can be assigned corresponding to the colours developed as per the colour chart enclosed to ZYM API system. Zero corresponds to a negative reaction; 5 to a reaction of maximum intensity. Values 1 through 4 are intermediate reactions depending on the level of intensity (weak). The colours remain stable for several hours after the strip has been inoculated with the reagents.

The ZYM API system contains the following enzymes. Alkaline phosphatase (substrate: 2-naphthyl phosphate pH 8.5); esterase C₄ (substrate: 2-naphthyl butyrate pH 7.1); esterase lipase C₈ (substrate: 2-naphthyl caprylate pH 7.1); lipase C₁₄ (substrate: 2-naphthyl myristate pH 7.1); leucine aminopeptidase (substrate: L-leucyl-2-naphthylamide pH 7.5); valine aminopeptidase (substrate: L-valyl-2-naphthylamide pH 7.5); cystine aminopeptidase (substrate:

Table I
*Strains of rapidly growing
scotochromogenic mycobacteria used in this study*

Species	Abbreviation	Strain numbers	Recognized strains and their code numbers*
<i>M. phlei</i>	PH	01-15	01, SN 101
<i>M. gilvum</i>	GI	01-05	01, Stanford 392 02, Stanford 35 = NCTC 10472** 03, Stanford 132 04, Stanford 205 05, Stanford 391
<i>M. duvalii</i>	DU	01-04	01, Stanford 51 = NCTC 8645 02, Stanford 70 = NCTC 358** 03, Stanford 579 = NCTC 509 04, Stanford 580 = NCTC 514
<i>M. vaccae</i>	VA	01	01, SN 918
<i>M. rhodesiae</i>	RH	01-03	01, ATCC 27024**
<i>M. gadium</i>	GM	01	01, ATCC 27726**
<i>M. flavescens</i>	FL	01	01, NCTC 10270
<i>M. agri</i>	AG	01	01, ATCC 27407**
<i>M. aurum</i>	A	01-06	01, ATCC 25790 = NCTC 10440**
<i>M. parafortuitum</i>	PA	01-09	01, ATCC 19686 = NCTC 10411**
<i>M. valentiae</i>	VT	01-05	01, ATCC 29356**
<i>M. neoaurum</i>	NE	01	01, ATCC 25795 = NCTC 10818**
<i>M. chubuense</i>	CH	01	01, ATCC 27278 = NCTC 18819**
<i>M. aichiense</i>	AI	01	01, ATCC 27280 = NCTC 10820**
<i>M. tokaiense</i>	TO	01	01, ATCC 27282 = NCTC 10821**

*ATCC, American Type Culture Collection, Rockville, Maryland, USA; NCTC, National Collection of Type Cultures, London; Stanford, Dr. J. L. STANFORD, Middlesex Hospital Medical School, London, England; SN, Dr. I. TARNOK, Forschungsinstitut Borstel, West Germany; Dr. M. TSUKAMURA, Chubu Chest Hospital, Obu, Aichi-ken, Japan. Other numbers are laboratory codes

**Type (paratype or co-type)

L-cystyl-2-naphthylamide pH 7.5); trypsin (substrate: N-benzoyl-DL-arginine-2-naphthylamide pH 8.5); chymotrypsin (substrate: N-benzoyl-DL-phenylalanine-2-naphthylester pH 7.1); acid phosphatase (substrate: 2-naphthyl-phosphate pH 5.4); phosphoamidase (substrate: naphthol-AS-BI-phosphodiamide pH 5.4); alpha-galactosidase (substrate: 6-Br-2-naphthyl-alpha-D-galactopyranoside pH 5.4); beta-galactosidase (substrate: 2-naphthyl-beta-D-galactopyranoside pH 5.4); beta-glucuronidase (substrate: naphthol-AS-BI-glucuronic acid pH 5.4); alpha-glucosidase (substrate: 2-naphthyl-alpha-D-glucopyranoside pH 5.4); beta-glucosidase (substrate: 6-Br-2-naphthyl-beta-D-glucopyranoside pH 5.4); beta-glucosaminidase (substrate: 1-naphthyl-N-acetyl-beta-D-glucosaminide pH 5.4); alpha-mannosidase (substrate: 6-Br-3-naphthyl-alpha-D-mannopyranoside pH 5.4); and alpha-fucosidase (substrate: 2-naphthyl-alpha-fucopyranoside pH 5.4).

Results and discussion

Results obtained with the ZYM API system are listed in Table II.

M. parafortuitum, *M. aurum* and *M. neoaurum* may be regarded as a complex [9]; they are differentiated with the alkaline phosphatase, lipase C₁₄, leucine aminopeptidase, valine aminopeptidase, acid phosphatase, alpha-glucosidase and beta-glucosidase.

M. gilvum differs from *M. duvalii* in leucine aminopeptidase, acid phosphatase and phosphoamidase.

M. valentiae differs from *M. rhodesiae* in acid phosphatase, alpha-glucosidase, beta-galactosidase and phosphoamidase.

M. vaccae differs from *M. aurum* in alpha-glucosidase, cystine aminopeptidase and phosphoamidase.

M. chubuense differs from *M. aichiense* in acid phosphatase, and from *M. tokaiense* in lipase C₁₄, alkaline phosphatase, acid phosphatase, valine aminopeptidase, cystine aminopeptidase, alpha-glucosidase and beta-glucosidase.

M. phlei differs from *M. agri* in valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, alkaline phosphatase and acid phosphatase.

M. gadium differs from *M. flavescens* in alkaline phosphatase, esterase lipase C₈, lipase C₁₄, valine aminopeptidase, cystine aminopeptidase and phosphoamidase.

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Table II

Results obtained with the API ZYM system with rapidly growing scotochromogenic mycobacteria

	Species of rapidly growing scotochromogenic mycobacteria														
	PH	GI	DU	VA	RH	GM	FL	AG	A	PA	VT	NE	CH	AI	TO
Alkaline phosphatase	+	+	+	++	+	—	++	—	—	—	+	++	+	+	—
Esterase C ₄	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Esterase lipase C ₈	++	+	—	++	++	—	++	++	++	++	++	++	++	++	++
Lipase C ₁₄	++	+	—	+	—	—	++	+	+	—	—	++	—	—	+
Leucine aminopeptidase	++	—	—	++	+	++	++	+	++	+	+	++	++	++	++
Valine aminopeptidase	++	—	—	—	—	—	+	—	—	—	—	+	—	—	+
Cystine aminopeptidase	++	—	—	—	—	—	+	—	+	—	—	+	—	—	+
Trypsin	++	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Chymotrypsin	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Acid phosphatase	++	+	—	—	++	—	—	—	—	—	—	+	+	++	—
Phosphoamidase	++	—	—	—	—	—	—	+	+	+	—	+	—	—	—
Alpha-galactosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Beta-galactosidase	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—
Beta-glucuronidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Alpha-glucosidase	—	—	—	—	++	—	—	—	++	—	—	—	—	—	+
Beta-glucosidase	—	—	—	—	—	—	—	—	—	—	—	+	—	—	+
Beta-glucosaminidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Alpha-mannosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Alpha-fucosidase	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

++: enzyme activity strong
 +: enzyme activity weak
 —: enzyme activity negative

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ELIMINATION OF F'LAC PLASMID BY DIFFERENT PSYCHOTROPIC DRUGS AND SOME RELATED COMPOUNDS

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Desipramine, trimipramine, protriptyline, noxyptyline, promazine, trimeprazine, triflupromazine and chlorprothixene methiodide eliminated the F'lac plasmid of *Escherichia coli*, while thiazinane, toluidine blue, lidocaine and procaine were ineffective in this respect. The plasmid eliminating action of the drug ceased in the presence of 0.05 M magnesium sulphate. Methylene blue did not inhibit plasmid elimination by the psychotropic drugs, and in presence of the dye even lidocaine and procaine became effective. Based on plasmid elimination in the presence of methylene blue and on the selective effects of the lon⁻ mutant, the plasmid eliminating mechanism of psychotropic drugs seems to differ from that of acridine orange.

The discovery of the curing effect of acridine orange [1, 2] and of ethidium bromide [3] initiated research on plasmid elimination. Acridine orange [4] and ethidium bromide [5] are able to intercalate in the DNA and this mechanism is believed to be responsible for their plasmid eliminating action, though the two compounds become inserted in different manners. Acridine orange intercalates between the broad furrows of the DNA, the binding site of DNA polymerase, while ethidium bromide between the narrow furrows, the site of RNA polymerase [6]. Nevertheless, both acridine orange [7] and ethidium bromide [8] inhibit DNA polymerase. Methylene blue with a planar structure similar to that of acridine orange becomes intercalated [9] and inhibits DNA polymerase [10] but the dye has no plasmid eliminating activity [1, 11, 12]. On the other hand, plasmid elimination by chlorpromazine, compound related to methylene blue, has been described by two research teams independently [13, 14].

Although the intercalation of chlorpromazine in the DNA [16] and its inhibitory action on DNA polymerase [16] has been recognized, studies on the plasmid eliminating action of chlorpromazine and its related compounds [17–19] could not prove the exclusive role of these effects in plasmid elimination by the phenothiazines. Thus e.g. promethazine does not inhibit DNA polymerase [20] but has a plasmid eliminating property [18].

The plasmid eliminating action of the tricyclic psychotherapeutic agents is independent of their intercalating property, as proved also by the fact that certain chlorpromazine metabolites fail to eliminate the plasmid, whereas antidepressants of three dimensional structure exert a plasmid curing action [11, 21].

Pharmacokinetic studies have directed attention to some relationships between molecular structure and the biological effect, among them to the cationic radical formation of antidepressants and tranquillizers [22] and to the role of the charge and space requirement of the side-chains [23].

It follows that the pharmacological effects of tricyclic amines are related partly to the ring system, partly to the space filling and charge of the substituent.

The purpose of the present work was to clarify to what extent do structural changes determining the pharmacological effect influence the plasmid eliminating activity of drugs. Four groups of compounds were tested. (1) A group of antidepressants with three dimensional configuration containing secondary and tertiary amine side-chains. (2) A group of phenothiazines of planar structure with different substituents but with a side-chain containing tertiary amine. (3) Phenothiazines with varying charge and space filling side-chains. (4) Tertiary amine substituted local anaesthetics that have a known membrane effect with and without dikationic side-chain.

Materials and methods

Compounds. Group 1. Desipramine (Pertofran, Ciba-Geigy, Basel); trimipramine (Sapient, EGYT, Budapest); protriptyline (Merk, Sharp and Dohme, Rahway, N.J.); nortriptyline (Agedal, Bayer, Leverkusen): Fig. 1.

Group 2. Chlorprothixine (Taractan, Hoffmann-La Roche, Basel); promazine (Sparine, Rhone Poulenc, Paris); triflupromazine (Vespin, Squibb, Middlesex); Trimeprazine (Rhone Poulenc, Paris): Fig. 2.

Group 3. Profenamine (Phenopropazine, Rhone Poulenc, Paris); thiazinanium (Multergan, Specia, Paris); toluidine blue (Merck); chlorpromazine methiodide (a gift from Dr. S. FÖLDEÁK, Department for Organic Chemistry of József Attila University, Szeged): Fig. 3.

Group 4. Lidocaine, procaine, methylene blue: Fig. 4.

Bacterial strain. *Escherichia coli* K12 LE 140 (T_6^R , T_1^S , Sm^R , lac^- Δ , Su^- , λ^R , Mal^-).

Culture media. MTY liquid and MTY agar media were used for cultivation and for the determination of live cell counts in the cultures [24], eosin-methylene blue (EMB) culture medium [25] for the differentiation of lac-negative bacterium colonies.

Elimination of the lac plasmid. The method described earlier was applied [18]. From the overnight preculture of *E. coli* K12 LE 140, a 10^{-4} dilution was prepared and 0.05 ml (approximately 5×10^3 cells) aliquots were inoculated into 5.0 ml MTY bouillon. Then varying concentrations of the plasmid eliminating compounds were given to the tubes and incubated at 37 °C for 24 hr. Different dilutions were made from the tube cultures showing bacterial growth and 0.1 ml each of the solutions was spread on EMB agar. The plates were incubated at 37 °C for 24 hr, then the lac-positive (plasmid carrying) and the lac-negative (plasmid lost) colonies were counted.

Results

Prior to the studies on plasmid elimination by the psychotropic drugs, their minimal bacteriostatic concentration (MIC) was determined. MTY culture medium 5 ml in volume was used; each volume contained the drug under study in concentrations ranging from 0–1000 $\mu\text{g/ml}$. From the *E. coli* K12 LE 140 culture, 5×10^3 cells were inoculated into the culture medium complemented with the drug, thus the basic cell number was approximately $1 \times 10^3/\text{ml}$.

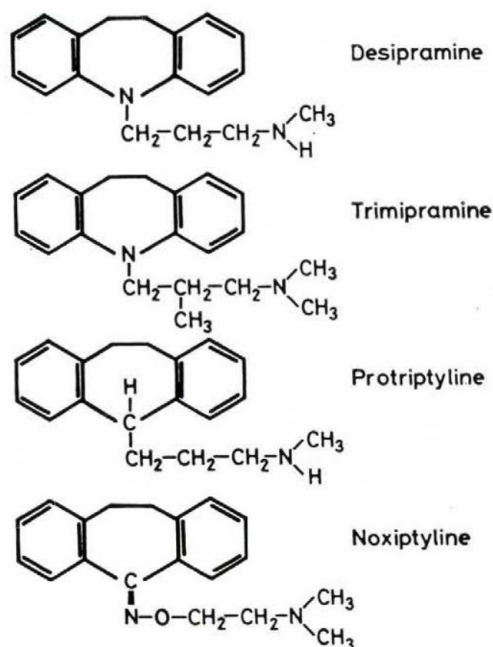


Fig. 1. Group 1 compounds

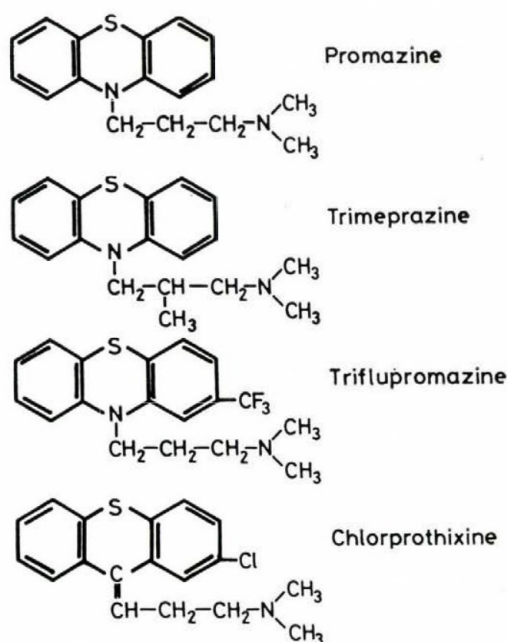


Fig. 2. Group 2 compounds

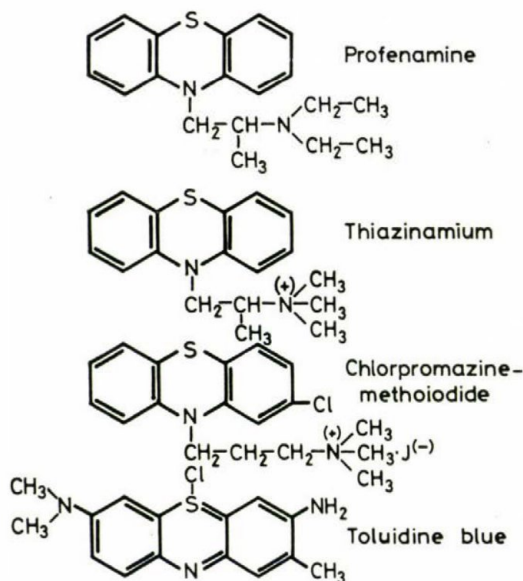


Fig. 3. Group 3 compounds

The samples were incubated at 37 °C for 24 hr, bacterial growth was examined in all the tubes containing different amount of the drug. The first tube showing no bacterial growth represented the minimal bacteriostatic concentration of the given drug (Table I). From among the antidepressants with a three dimensional structure, desipramine and protriptyline containing secondary amine side-chains showed lower MIC values than did trimipramine and nioxip-tyline possessing tertiary amine side-chains. Among the compounds of Group 2, triflupromazine substituted at the second position of the ring system had

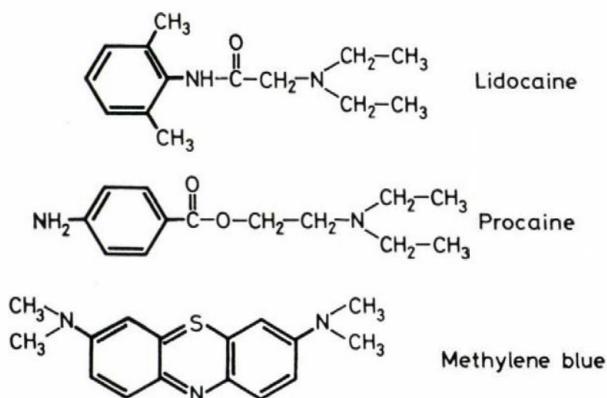


Fig. 4. Group 4 compounds

a bacteriostatic effect at lower concentrations that had promazine. The least effective was trimeprazine, with a 150 $\mu\text{g/ml}$ MIC value. Among the substituted phenothiazines in Group 4, chlorpromazine methiodide inhibited bacterial growth at higher concentrations than did chlorpromazine. From among the compounds of Group 4, in the case of profenamine and thiazinanium, changes in space filling and charge of the substituted side-chains led to a weakening of the bacteriostatic effect while in the case of toluidine blue, charge asymmetry of the ring system caused the lowering of the bacteriostatic effect and the elevation of the MIC value. Of the two local anaesthetics, lidocaine demonstrated a very weak antibacterial activity, and even 20 mg/ml of procaine was not capable of inhibiting growth (Table I).

Studies at sub-inhibitory concentrations of the agents revealed that from among the antidepressants, the secondary amine substituted desipramine and protriptylene eliminated F⁺lac plasmid at lower concentrations than did the tertiary amine substituted alimemazine and noxipityline. The majority of phenothiazines eliminated the plasmid with a frequency similar as the antidepressants (Table II). An exception was thiazinanium possessing a quaternary amino group, where the assymetrically substituted chlorpromazine methiodide

Table I

Minimal bacteriostatic concentrations for E. coli K12 LE 140

Compounds	MIC $\mu\text{g/ml}$
Desipramine	70
Trimipramine	200
Protriptyline	80
Noxipityline	300
Promazine	90
Trimeprazine	150
Triflupromazine	80
Chlorprothixine	50
Profenamine	230
Thiazinanium	230
Chlorpromazine methiodide	180
Toluidine blue	280
Lidocaine	3500
Procaine	> 20000

Table II
Plasmid eliminating action of various psychotherapeutics

Compounds	Concentration $\mu\text{g/ml}$	Plasmid elimination per cent	Colonies examined
Desipramine	50	23.4	3600
Trimipramine	180	25.8	5700
Protriptyline	50	22.9	4400
Noxiptyline	260	41.0	3300
Promazine	60	40.0	6950
Trimeprazine	130	22.0	4700
Trifluopromazine	60	25.0	3500
Chlorprothixine	40	87.0	3450
Profenamine	220	54.0	7100
Thiazinanium	190	0.0	5000
Chlorpromazine methoiodide	160	240.0	2900
Toluidine blue	240	0.0	3500
Lidocaine	2950	0.3	4360
Procaine	18000	0.0	4020

did not suspend plasmid elimination by the compound. Several factors are probably involved in the differing effect of thiazinanium and chlorpromazine methoiodide, so e.g. the distance between the two nitrogen atoms in the dicationic side-chain (4.5 Å for thiazinanium, 6.0 Å for chlorpromazine methoiodide), further the space requirement of the side-chain (thiazinanium isopropyl-, chlorpromazine methoiodide propyl-). The two small molecular tertiary amines lidocaine and procaine, showed no plasmid eliminating effect, although lidocaine has a dicationic side-chain.

The plasmid eliminating effect of the compounds was definitely reduced in the presence of magnesium ions, despite the fact that twofold of the minimal bacteriostatic concentration was used in the tests (Table III). These results are in agreement with earlier observations indicating that plasmid elimination by chlorpromazine, imipramine, acridine orange and ethidium bromide is suspended in the presence of bivalent metal ions [19].

HIROTA [1] observed that the in itself ineffective methylene blue, as a structural analogue, inhibits plasmid elimination by acridine orange. Based

Table III

Plasmid eliminating action of positively charged therapeutic drugs and local anaesthetics in the presence of 0.05 M MgSO₄

Compounds	Plasmid elimination per cent	Concentration $\mu\text{g/ml}^*$	Colonies examined
Desipramine	0.1	140	6476
Trimipramine	0.0	400	5908
Protriptyline	0.4	160	4835
Noxipityline	0.9	600	6909
Promazine	0.3	180	8237
Trimeprazine	0.3	300	5774
Triflupromazine	0.3	160	5525
Chlorprothixine	0.2	100	7575
Profenamine	0.4	480	6580
Thiazinanium	0.0	460	6665
Chlorpromazine methoiodide	0.1	360	6925
Toluidine blue	0.0	440	5250
Lidocaine	0.0	7000	5480
Procaine	not tested	not tested	

*Concentrations used for plasmid elimination are double the MIC values

on this finding we tested the plasmid eliminating action of some representative compounds in the presence of the dye in order to support or also to exclude plasmid elimination by the psychotherapeutics *via* the acridine orange mechanism. It was found that methylene blue somewhat increased the plasmid eliminating action of the drugs. The synergistic effect was most marked with lidocaine (Table IV). These data indicate that the condensed three rings of methylene blue and the tertiary amine substituted side-chain of lidocaine and procaine together inhibit the replication of the F⁺lac plasmid. The synergism of the two compounds ineffective in themselves suggests that plasmid replication has at least two vulnerable points at which the compounds with dicationic side-chains exert their action.

We could establish that the optimal biological effect ensues at pH values above 7, therefore in the first step, the amino group responsible for the binding has to be of basic character. The effect of the compound on the cell membrane is also shown by the fact that in their presence lon⁻ mutants become selected with various frequency (Table V).

The bactericidal effect of the compounds was tested in subcultures prepared from lactose positive and lactose negative colonies. The lac-negative and lac-positive cells were identical in sensitivity, whereas the lon⁻ mutants exhibited a moderate resistance. Neither of the two local anaesthetics did select lon⁻ mutants, even in the presence of methylene blue.

Table IV

Plasmid eliminating potency of psychotherapeutic drugs and local anaesthetics in the presence of 100 µg/ml methylene blue

Compounds	MIC µg/ml	Plasmid elimination		Colonies examined
		frequency per cent	concentration, µg/ml	
Desipramine	60	44.0	5á	5200
Trimipranime	200	18.7	180	5640
Triflupromazine	80	25.6	60	6108
Chlorprothixine	60	57.2	50	4950
Lidocaine	1150	23.9	1040	6430
Procaine	6800	5.5	6500	8560
Thiazinanium	220	1.7	180	2490

Table V

Selection of lon⁻ mutants under the effect of different compounds

Compounds	Concentration, µg/ml	lon ⁻ mutant, per cent	Colonies examined
Desipramine	60	0.54	3600
Trimipramine	180	0.94	5700
Protriptyline	60	2.00	4400
Noxyptyline	270	1.50	3300
Promazine	70	0.40	6950
Trimeprazine	150	2.10	4700
Triflupromazine	70	1.70	3500
Chlorprothixine	30	1.40	3450
Profenamine	210	0.30	7100
Thiazinanium	150	0.00	5000
Chlorpromazine methoiodide	170	0.50	2900
Toluidine blue	260	0.00	3500
Lidocaine	2950	0.00	4360
Procaine	18000	0.00	4020

Discussion

Of the known plasmid eliminating mechanisms, the simplest is the destruction of bacteria carrying autonomous plasmid in the presence of SDS [26] or that of the integrated plasmid carrying organisms in the presence of sodium cholate [27]. A detergent effect is the common trait of the two compounds. The other mechanism of plasmid elimination is based on the intercalation in the DNA of different, usually tricyclic, macromolecules. Since the plasmid carrying and plasmid deprived cells showed an identical sensitivity to the agents under study, a selective mechanism similar as that of SDS [26] or cytarabine [28] could be excluded. Neither is the intercalation in the DNA of some drugs an unequivocal question, because antidepressants of three-dimensional structure do not become intercalated due to the lack of planarity, whereas the phenothiazines with planar structure become inserted [15].

Beside plasmid elimination, antidepressants and phenothiazines have the common property of binding to membrane components [29]. The antidepressants possessing a secondary amine side-chain are more effective in inhibiting bacterial growth and in eliminating the plasmid than are the drugs with tertiary amine side-chains, whereas the plasmid eliminating potency of quaternary amines is weaker and it is believed that the quaternary methyl derivative of chlorpromazine is unable to penetrate across the cell membrane [30]. Several data indicate the binding of antidepressants and phenothiazines to the cell membrane. Chlorpromazine and imipramine may become attached through their cationic amine group to the phospholipids of the cell membrane [31, 35], the other part of the molecule becomes intercalated between the fatty acid chains of the membrane [32, 33]. This would indicate that the plasmid eliminating ability of a given compound is the function of a membrane/buffer distribution coefficient. This coefficient is 1700 for chlorpromazine, 295 for imipramine and only 17 for lidocaine [34].

The effect of the drugs on the cell membrane is supported also by the finding that in the presence of bivalent cations their action can be reversed [34, 19].

It has been recognized that these drugs alter the fluidity of membrane lipids [36], while inducing a rigidity to certain lipids [37, 38]. Structural changes similar to those observed on the eukaryotic cell membrane might ensue also on the prokaryotic membrane and this change in cell membrane structure, the site serving for plasmid replication, may lead to its cessation.

The appearance of great numbers of lon⁻ mutants under the effect of antidepressants and phenothiazines is the result of selection. The same has been shown earlier for chlorpromazine, promethazine [39] and imipramine [21]. Many of the psychotropic drugs with a tricyclic amine basic structure exert a lon-selective effect, whereas in the presence of acridine orange and ethidium

bromide no lon⁻ mutants can be obtained [21, 39]. This selective mechanism shows a certain analogy with the appearance of haemin deficient mutants that become selected in the presence of amino glycosides [40, 41].

The latter hypothesis is supported by the finding that in the presence of chlorpromazine the rate of unsaturated fatty acids decreases in the bacterial cells and their lipid composition is altered as a consequence of adaptation to the drug [42].

Since the effect of the tricyclic amines is related their lipid solubility, the decrease in adaptive lipid content may have the result that the cells able to bind less of the drugs will survive at the expense of those with normal lipid content. Small numbers of lon⁻ mutants probably arise in bacterial populations by spontaneous mutation and the mutants excrete cholanic acid to the surface of the cells. On the other hand, the cholanic acid inhibits the binding to the membrane of tricyclic amines and the development of the cell-impairing effect. Changes in bacterial staining also indicate that the antibacterial effect of the drugs is based on membrane impairment [17]. Selection of lon⁻ mutants and the plasmid eliminating action represent a newly discovered common property of a large group of psychoactive drugs.

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UROPORPHYRINOGEN III COSYNTASE-DEFICIENT MUTANT OF BACILLUS SUBTILIS

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A new type of porphyrin-requiring mutant was isolated by streptomycin selection from *Bacillus subtilis* strain 168 trpC2. The mutant accumulated large quantities of uroporphyrin I and coproporphyrin I. A cell free extract transformed δ -aminolaevulinic acid to uroporphyrin I and coproporphyrin I; when it was incubated together with an extract of uroporphyrinogen I synthase-deficient strain, protoporphyrin was formed. These data prove that the isolated mutant was uroporphyrinogen III cosynthase-deficient.

There are numerous porphyrin-requiring bacterial mutants [1–4] but uroporphyrinogen (UROGEN) III cosynthase-deficient mutants have been described only recently [5, 6]. UROGEN III cosynthase-deficient mutant of *Salmonella typhi-murium* was first characterized by SASARMAN and DESROCHERS [5]. A mutant with a similar deficiency has probably been demonstrated in *Escherichia coli* [6], but this is known only to accumulate uroporphyrin (URO) I and coproporphyrin (COPRO) I.

A number of theories have been put forward to explain the porphobilinogen (PBG) $\rightarrow$ UROGEN III transformation [7]. Two enzymes are necessary for this transformation: UROGEN I synthase and UROGEN III cosynthase. Without UROGEN III cosynthase, UROGEN I is formed, which is converted to COPROGEN I under the action of the decarboxylating enzyme. Here the synthesis stops; UROGEN is not formed from PBG without UROGEN I synthase; the cosynthase alone is ineffective.

With streptomycin selection we have succeeded in isolating a UROGEN III cosynthase-deficient mutant from *Bacillus subtilis*. In the present paper, some of the characteristics of this mutant are described. A report of the mapping of the gene of UROGEN III cosynthase is under publication.

Materials and methods

Strains. The porphyrin mutants of *B. subtilis* 168 used in this work are listed in Table I.

Growth media. A chemically defined medium (gGM) [1] was supplemented with tryptophan (50 μ g/ml). In the case of porphyrin auxotrophs, HAC (gGM supplemented with tryptophan, 2.5 μ g of haemin per ml, 50 μ g of cysteine per ml, and 1 to 2 mg of bovine serum albumin per ml) or HA (HAC minus cysteine) medium was used [8]. Agar media contained 1.5% agar (Difco).

Table I
Strains examined

Strain	Previous designation	Genotype	Enzyme defect in porphyrin biosynthesis	Supplied by
Sz 1	RI 2	<i>trpC2</i>	None	P. SCHAEFFER
Sz 3	RI 5	<i>trpC2 hemA1</i>	ALA synthetase	T. ANDERSON
Sz 15	I/1	<i>trpC2 hemB1</i>	ALA dehydrase	I. BEREK
Sz 16	II/33	<i>trpC2 hemC33</i>	UROGEN I synthase	I. BEREK
Sz 34	—	<i>trpC2 hemD11</i>	UROGEN III cosynthase	

Extraction of porphyrins. Bacteria were grown in HAC medium at 37 °C under continuous shaking. After reaching OD 0.5 at 620 nm, the culture was centrifuged, washed in gGM medium and centrifuged again. The pellet was resuspended in the same volume of gGM (with tryptophan) and grown for 4 hr. This manipulation was necessary to eliminate the haemin which had not been used up in order to prevent a feed-back inhibition of haem synthesis. The cultures were centrifuged and the bacterial pellet as well as the supernatant were treated separately as described earlier [2].

Chromatography. Porphyrins were separated and measured as described previously [2, 9]. URO and COPRO isomers I and III were identified by the methods of FALK and BENSON [10] and YUAN and RUSSELL [11].

Porphyrin synthesis in extracts of bacteria. The strains were grown in HAC medium. Cultures in the log phase (for about 5 hr) were centrifuged; the bacteria were sonicated (MSE Ultrasonic Unit Model 150 W at 12 μ amplitude for 3 $\times$ 2 min at 0 °C), and the solutions were centrifuged. The reaction mixture contained 3 ml of various extracts (15 mg of protein/ml in 0.1 M Tris pH 8.2), 1.5 μ M δ -aminolaevulinic acid and 0.5 μ M methionine. Incubation was done at 30 °C, under shaking for 4 hr. This method was a modification of that by SASARMAN *et al.* [3, 5]. Porphyrins synthesized by the extracts were extracted by ethyl acetate-acetic acid [12].

Results

Physiology of the mutant Sz 34. To a certain extent, this mutant differs from the other porphyrin mutants. Similarly to the mutants bearing *hemA*, *hemB* and *hemC* loci, on haemin-containing synthetic medium methionine or cysteine is required for growth, although this requirement is less strict. In contrast to the *hemB* and *hemC* mutant, δ -aminolaevulinic acid is not accumulated, whereas porphyrins are; in UV light the colonies fluoresced characteristically. The intracellularly and extracellularly accumulated porphyrins were determined (Table II). The final intermediate detectable was COPRO; protoporphyrin could not be demonstrated. In all cases it was the I isomer that had accumulated. This result strongly suggests that the enzyme UROGEN III cosynthase is deficient in the mutant (Fig. 1).

Porphyrin synthesis in extracts of the mutants. The results are listed in Table III. The Sz 16 mutant accumulated PBG, owing to the deficiency of UROGEN I synthase. If cell-free extracts of Sz 16 and Sz 34 are mixed, porphyrin synthesis takes place, isomers III being obtained from URO and COPRO

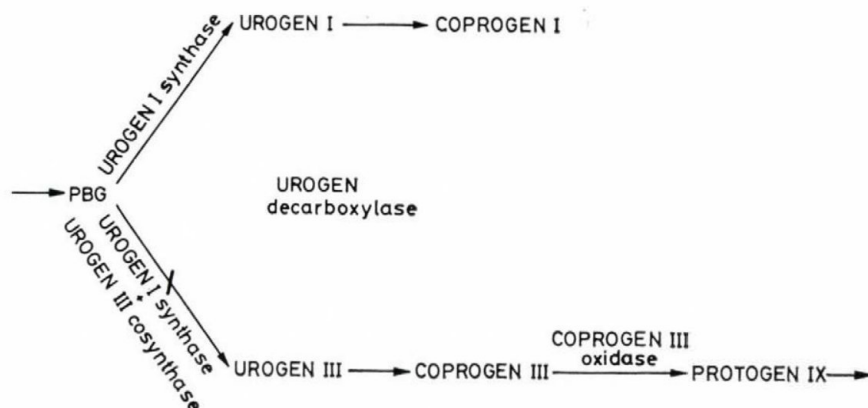


Fig. 1. Enzymes of the porphyrin-biosynthetic pathway. Crossed arrow indicates block in the synthesis

in the amounts shown in Table III. These data confirm that the Sz 34 mutant is UROGEN III cosynthase deficient.

Table II

Amount of porphyrin intermediates accumulated by mutant Sz 34

	Intermediates*						
	8-COOH	7-COOH	6-COOH	5-COOH	4-COOH	3-COOH	2-COOH
Intracellular	34.27	6.55	8.06	9.57	25.20	0**	0
Extracellular	40.60	8.12	18.56	30.16	30.16	0	0

*The values refer to μg porphyrin/g bacteria (wet weight); porphyrin intermediates containing 8-COOH(URO), 7-COOH, 6-COOH, 5-COOH, 4-COOH(COPRO), 3-COOH, 2-COOH(PROTO) groups

**No detectable amount of the metabolite was found

Table III

Porphyrin synthesis from δ -aminolaevulinic acid in extracts of mutants

Extract	URO	COPRO	PROTO
Sz 16	0	0	0
Sz 34	69*	31*	0
Sz 16 + Sz 34	44	30	18

The values refer to nmole porphyrin

*I isomer

Discussion

The *B. subtilis* porphyrin-requiring mutants fall into two groups [13]. The UROGEN III cosynthase-deficient mutant differs in several of its properties from the other porphyrin mutants. URO I and COPRO I are accumulated in large amounts. When a cell-free extract is mixed with an extract of UROGEN I synthase-deficient mutant, normal porphyrin synthesis occurs. The gene (*hemD*), responsible for UROGEN III cosynthase is linked to the *hemA*, *hemB* and *hemC* loci and is distant from the other mapped *hem* genes [14].

A study of the mutant described may shed light on the formation of URO III, in connection with which more than 20 theories are known. There are also numerous conceptions regarding the branching site of vitamin B₁₂ synthesis and porphyrin synthesis [15, 16]. Our preliminary examinations show that this mutant is not or to a negligible extent able to produce corrinoids. In human congenital porphyria too, URO I and COPRO I are accumulated [17]. The mutants may be of assistance in research into that disease.

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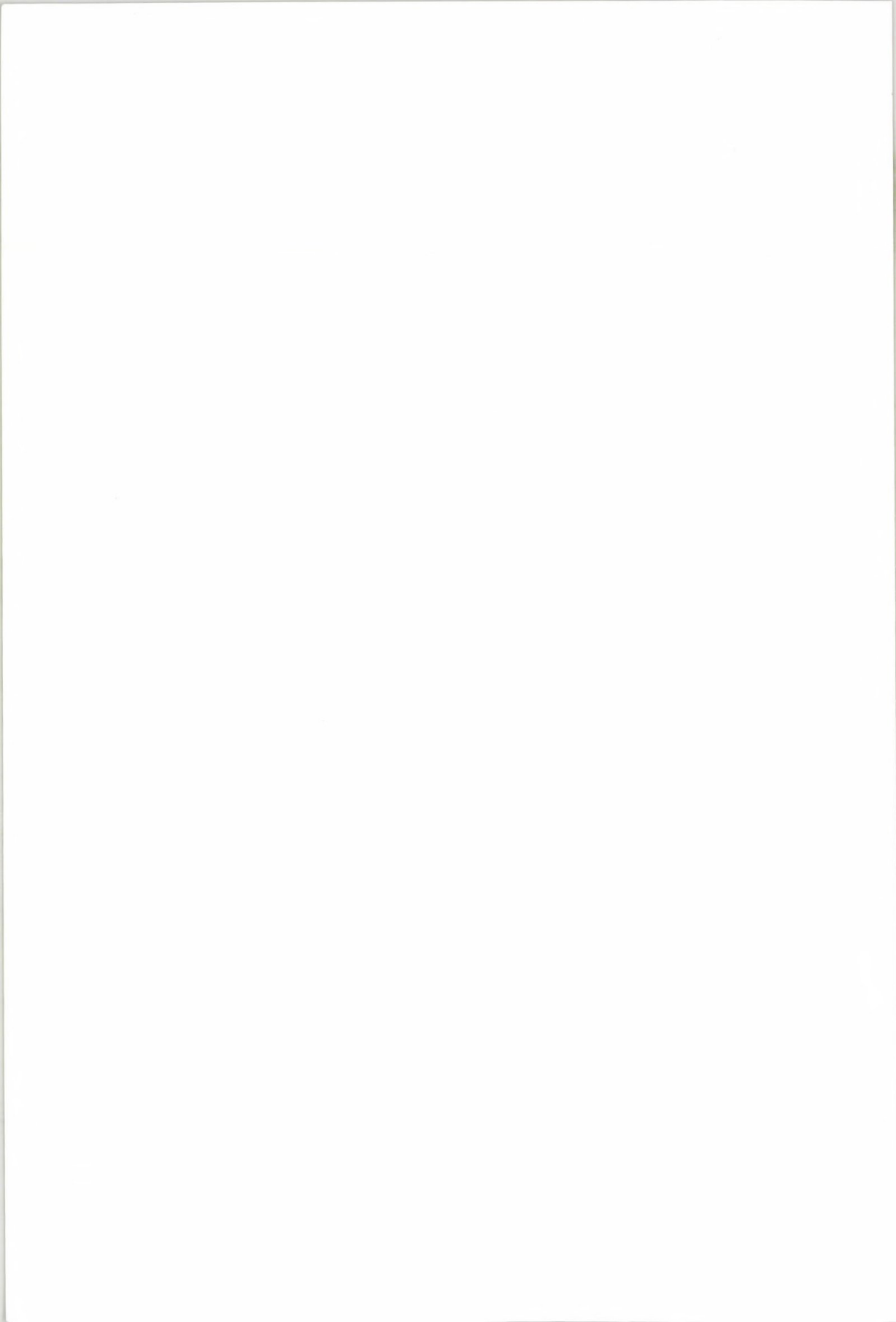
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